Chris A. Wozniak
Alan McHughen *Editors*

Regulation of Agricultural Biotechnology: The United States and Canada

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Foreword

When governments first began approving genetically engineered crops (called GMOs) for commercial production and consumption in the mid-1990s, the technology had not yet become a lightning rod for political controversy. European regulators initially approved GMOs nearly as readily as regulators in the United States and Canada. Then when a political campaign against GMOs began after 1996, European regulatory systems became more highly precautionary and by 1998 new approvals were suspended completely. Fortunately, both the United States and Canada managed to avoid the full regulatory blockage that continues to hamper the technology in Europe.

This excellent new volume edited by Chris Wozniak and Alan McHughen maps in considerable detail the legal and institutional terrain governing agricultural biotechnology regulation in both the United States and Canada. Most of the chapter authors are either regulators themselves or academic specialists comfortable with the legal and technical thinking of regulators. If you want to learn how GMO crops – and animals – are seen by regulators in the United States and Canada, and also by some of the applicants for regulatory approval, this is the book to read.

Wozniak and McHughen have chosen wisely to ground the volume in a clear understanding of how regulatory systems for agricultural GMOs emerged in the United States and Canada in the 1980s and early 1990s, before any crops were formally commercialized. In the United States, this meant the emergence of something called the Coordinated Federal Framework, which assigned separate roles to three existing agencies – the Environmental Protection Agency (EPA), the Department of Agriculture (USDA), and the Food and Drug Administration (FDA). The United States has stood nearly alone in deciding to regulate this new technology without creating new laws or new institutions. Canada, meanwhile, created a separate regulatory trigger for the environmental release of what it called Plants with Novel Traits (PNTs). It is of interest that both approaches have managed to function, even in the face of intense popular and political controversy over GMOs. Understanding the sources of this North American success is one subtext of the Wozniak and McHughen volume.

Even the most experienced specialist will find new things to learn in this book. Chapters are included on the regulation of microorganisms, on the symbiotic control

of Pierce's disease, on the regulated management of insect resistance to plant-incorporated protectants, and on the regulation of genetically engineered animals and insects. Important distinctions of larger significance are also explored – for example, the distinction between regulatory science versus research science, versus safety assessment. Regulatory costs to applicants are examined, and the role of public sector research in facilitating market access for GMO crops is explained.

So – just when you thought there was nothing new to say about GMOs, along comes this richly detailed and up to date collection. Whether you are an academic researcher studying regulations, an actual regulator hoping to understand your role in a more complete historical and cross-national context, or a technology developer trying to anticipate the regulatory hurdles you will face, this new book will be of considerable value.

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Preface

"*A book on Agricultural Biotechnology regulations? That seems oxymoronic.*" At first thought, perhaps it is. After all, a published book is as permanent as regulations are fleeting. Once a book is printed, any errors or omissions, even simple typos, are there for readers to spot and chuckle over for years to come.

In contrast, regulations can and do change quickly. It would take a virtual blog to keep up with the barrage of regulatory changes, considering the multitude of statutes, agencies and departments involved, not to mention the policy calls which influence the direction of oversight as well as enforcement and compliance matters.

So, knowing that regulations are subject to such rapid modification, why would anyone endeavor to compile a book on regulations – Why would anyone buy one – when the permanent book is destined to be out of date before the ink is dry?

While it is true that the minutia of regulations do evolve rapidly, the underpinning supports for the regulations do not. Regulations governing agricultural biotechnology in the USA, Canada, and, for that matter, even those of the European Union are founded on unmoving monoliths, essentially unchanged over the quarter century since the first products of the technology were developed. Scientific and regulatory analyses of the safety issues surrounding rDNA as applied to food and agriculture date back to the early and mid-1980s, including those from the Organisation for Economic Cooperation and Development (OECD 1982), US National Academies of Science (NAS 1983, 1987), US Office of Science and Technology Policy (OSTP 1986), and the Canadian Agricultural Research Council (CARC) in 1988. These scientific analyses were hypothetical and predictive, as they were conducted, for the most part, prior to actual field trial experience with genetically engineered plants, which only started in 1987–1988. In this respect, those studies have been remarkably prophetic, as the findings and recommendations have largely borne out with time and experience.

In this volume we strive to present and describe the underlying concepts supporting the US and Canadian regulatory structures, less so on the ephemeral, minute details. To that end we contacted authorities from US and Canadian government agencies, industry and academia to share their expertise so readers can benefit from their collective diverse perspectives in describing our regulatory structures. With the

regulatory conceptual framework thus provided, specific details may then be acquired from the various agency websites.

It is important to keep in mind that authors of the chapters contained in this volume are writing from their own perspective, which may be that of a government regulator, academic researcher, industry scientist, attorney or program administrator. It is this mix of viewpoints, some contrasting and some in harmony, which makes this compilation intriguing and historical. While the regulatory system for biotechnology in agriculture has often been perceived as static in nature and inflexible (i.e. written in stone!), this is far from the truth. In addition to the dry matter of regulations and statutes, you will find helpful information to aid in navigating the system, an indication of some of the potential pitfalls of those traversing the gauntlet of biotechnology regulation and suggestions on what can be done to improve this dynamic system.

Wozniak and McHughen

References

National Academy of Sciences (1983) Risk assessment in the federal government: managing the process. National Academies Press, Washington, DC

National Academy of Sciences (1987) Introduction of recombinant DNA-engineered organisms into the environment: key issues. National Academies Press, Washington, DC

Organisation for Economic Cooperation and Development (1982) Biotechnology: International trends and perspectives. OECD, Paris. http://www.oecd.org/science/biotechnologypolicies/2097562.pdf. Accessed 22 Nov. 2010

OSTP (1986) Coordinated Framework for Regulation of Biotechnology. 51 FR 23302-93, June 26, 1986. www1.usgs.gov/usbiotechreg/read_file.nbii. Accessed 22 Nov. 2010

Contents

1 An Introduction to Agricultural Biotechnology Regulation in the U.S. ... 1
Chris A. Wozniak, Annabel Fellman Waggoner, and Sheryl Reilly

2 Regulation of Genetically Modified Crops in USA and Canada: Canadian Overview ... 15
Stuart J. Smyth and Alan McHughen

3 Regulation of Genetically Modified Crops in USA and Canada: American Overview ... 35
Alan McHughen and Stuart J. Smyth

4 Regulation of Genetically Engineered Microorganisms Under FIFRA, FFDCA and TSCA ... 57
Chris A. Wozniak, Gwendolyn McClung, Joel Gagliardi, Mark Segal, and Keith Matthews

5 The Status, Promise and Potential Perils of Commercially Available Genetically Modified Microorganisms in Agriculture and the Environment ... 95
Anne K. Vidaver, Sue Tolin, and Angela Post

6 Regulatory Experiences in Symbiotic Control of Pierce's Disease ... 103
Thomas A. Miller

7 Over a Decade of Crop Transgenes Out-of-Place ... 123
Norman C. Ellstrand

8 The Regulation of Organisms Used in Agriculture Under the *Canadian Environmental Protection Act, 1999* ... 137
Heather Darch and Arash Shahsavarani

9 Regulating the Environmental Release of Plants with Novel Traits in Canada ... 147
Krista Thomas and Stephen Yarrow

10 EPA Regulatory Requirements for Plant-Incorporated Protectants ... 163
John L. Kough and Rebecca Edelstein

11 United States Environmental Protection Agency Insect Resistance Management Programs for Plant-Incorporated Protectants and Use of Simulation Modeling ... 175
Sharlene R. Matten, Robert J. Frederick, and Alan H. Reynolds

12 Development and Regulation of the *Plum Pox Virus* Resistant Transgenic Plum 'HoneySweet' ... 269
Ralph Scorza, Ann Callahan, Michel Ravelonandro, and Michael Braverman

13 Genetically Engineered Insects – Regulatory Progress and Challenges ... 281
Luke S. Alphey and Camilla J. Beech

14 Regulation of Genetically Engineered Animals ... 301
Elizabeth A. Maga and James D. Murray

15 Regulatory Science, Research Science and Innovation in Agricultural Biotechnology ... 317
Alan Raybould, Ryan Kurtz, and Lawrence Zeph

16 The Globalization of Agricultural Biotechnology: Implications for Regulatory Compliance, Stewardship and Stakeholder Engagement ... 335
Jeffrey D. Rowe, Firoz Amijee, Steven D. Brody, Greg G. Wandrey and Courtney Chabot Dreyer

17 Facilitating Market Access for GE Crops Developed Through Public Sector Research ... 377
Nina V. Fedoroff and Roger Beachy

Index ... 391

Chapter 1
An Introduction to Agricultural Biotechnology Regulation in the U.S.

Chris A. Wozniak, Annabel Fellman Waggoner, and Sheryl Reilly

Abstract The regulation of agricultural plant and microbial biotechnology products in the United States of America has a rich history that reflects the challenges the federal government has faced in the development of appropriate rules and standards needed to determine their safety to humans and the environment. Several factors – the insufficient global food supply, loss or curtailment of the use of older chemistries to control pests due to risks and environmental persistence, the rising demand for safer food commodities, and the uncertainty surrounding the sustainability of agriculture in this and other countries – have added to these challenges. The chapter introduces the U.S. Coordinated Framework for the Regulation of Biotechnology ("Framework"), and the roles of its members: the U.S. Department of Agriculture (USDA), the U.S. Food and Drug Administration (FDA), and the U.S. Environmental Protection Agency (EPA) in regulating agricultural biotechnology in accordance with U.S. federal statutes. The Framework agencies use scientific, risk-based approaches in carrying out their regulatory responsibilities for the products of biotechnology. Relying on their experiences with risk assessment and risk management policies and principles for more conventional products, the Framework agencies have adapted new risk and exposure scenarios into their evaluations to ensure the safe use of these products in agriculture.

Keywords Biotechnology • Federal Food, Drug, and Cosmetic Act (FFDCA) • Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) • Food Quality Protection Act (FQPA) • Genetically engineered crops • Plant-incorporated protectants • Plant Protection Act • Regulated articles • Regulation • Toxic Substances Control Act (TSCA) • U.S. Coordinated framework

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1.1 Background

Regulatory oversight of biotechnology has been in place in the United States (U.S.) since the 1970s, although early guidance documents did not truly have the regulatory teeth to adequately handle the oversight of all the organisms being engineered for research or commercial purposes (Pizzuli 1984). Since those early days, the regulatory system in the U.S. has developed and adapted as needed to regulate microbes, plants, fungi and animals as products of biotechnology for environmental release and commercialization. For example, genetically engineered (GE) crops have been rapidly adopted in the U.S. with about 94% of soybeans, 90% of cotton, 88 % of field corn, and 55 % of canola acreages being derived from rDNA techniques (ERS 2011; Fig. 1.1). The percent adoption of other GE crops, such as sugarbeet and alfalfa, has also increased with no evidence that this trend will not continue in the U.S. and elsewhere (ISAAA 2012). The significant adoption of GE crops reflects a functioning U.S. regulatory system.

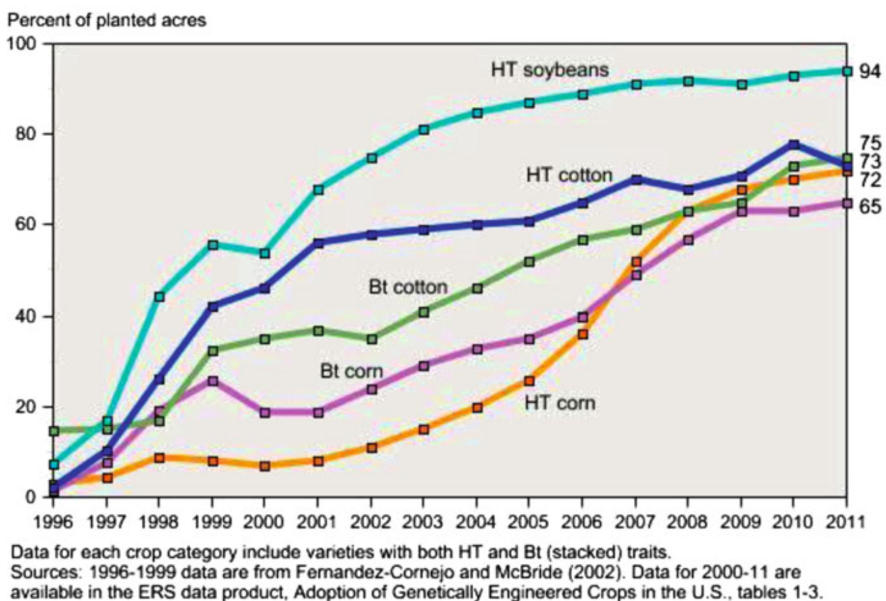

Fig. 1.1 Growth in adoption of genetically engineered crops continues in the U.S. *HT* = Herbicide tolerant, *Bt* = Expressing an insecticidal protein from *Bacillus thuringiensis*

The purpose of this chapter is to elucidate the historic guidance, regulations, and procedures that govern experimentation with genetically engineered (GE) organisms in experimental field trials and unconfined environmental release of GE organisms. The safety assessment of genetically engineered food will also be briefly discussed. All of the information for this analysis was obtained from publicly available sources provided by the respective regulatory authorities and the primary literature. The other focus of this document is the Coordinated Framework for Regulation of Biotechnology, a policy document regarding regulation of biotechnology products which was published by the Office of Science and Technology Policy (OSTP) in 1986 (OSTP 1986). The Framework involves key roles for the U.S. Environmental Protection Agency (EPA), the U.S. Department of Agriculture, Animal and Plant Health Protection Service (USDA-APHIS), and the U.S. Food and Drug Administration (FDA). This chapter restricts its focus to regulation of GE microbes and plants, while other chapters in this book examine regulation of insects and vertebrate animals.

While this book reflects the application of recombinant DNA (rDNA) to the genetic engineering of organisms, the term 'biotechnology' can be viewed more broadly to reflect the application of biology to man's needs and desires. This is especially important when making the distinction between 'genetically engineered' organisms and the more general term 'products of biotechnology' or 'genetically modified' (GM). It is worth noting that all crops plants domesticated for use by man have been modified genetically through selection and plant breeding practices. However, for the purposes of this chapter and the majority of this book, we will reserve the term as applicable to products and processes derived from the use of rDNA.

Microbial biopesticides have been regulated under FIFRA since 1948 (e.g., *Bacillus popillae*) and genetically engineered microbial pest control agents (MPCA) since the mid-1980s using the same statutory authority with regulations (40 CFR 158.2100) modified and updated over time (see Chap. 4 for more detail). It is important to note that the same regulations and data requirements were applied to both GE and non-GE MPCAs . With the advent of *in planta* expression of pesticidal substances in the late 1980s, thus creating plant-incorporated protectants (PIPs), regulations were again updated to reflect the novelty of these pesticides (EPA 1994, 2001b). Technological developments take time and regulations must remain dynamic and flexible in order to keep pace with the technology (Jepson 2003). This is certainly the case with agricultural biotechnology.

1.2 Early Regulatory Development for Biotechnology Products

For centuries, humans have improved crop plants through selective breeding and hybridization — largely through the controlled pollination of plants. Meiotic recombination following pollination that may include undesirable traits which have to be bred out of the new plant by multiple backcrosses before a hybrid can become a commercially viable new variety. In more recent times, plant breeders created new varieties using chemicals or irradiation to provide unique traits in

plants via mutagenesis. Plant transformation is a form of plant breeding with one very important difference — plant biotechnology allows for the transfer of specific genetic information from species related or unrelated to the plant with modifications to the expression pattern of these transgenes in both a temporal and spatial manner.

Traditional plant breeding involves the crossing of thousands of genes, whereas plant biotechnology allows for the transfer of only one or a few desirable genes.

Responding to the rapid increase in the production of biotechnology products, there was a realization of the need for some sort of guidance to ensure that public health and the environment are adequately protected from the potential risks of this technology. As products began moving from the laboratory toward the market, scientists and regulatory agencies realized that there should be regulatory mechanisms to ensure that these new products did not adversely affect public health or the environment (Howland 1987). To clarify regulatory jurisdiction over biotechnology products, the Reagan Administration established an interagency working group under the White House Cabinet Council on Natural Resources and the Environment (now known as the Domestic Policy Council) in 1984 and the Biotechnology Science Coordinating Committee in 1985 (Patterson and Josling 2001). The working group's principle goal was to ensure the regulatory process adequately considered health and environmental safety consequences of the products of biotechnology as they move from the laboratory to the marketplace. Safety was not their only concern; however, as the Council also emphasized the importance of not stifling innovation or enervating the competitiveness of the U.S. biotech industry. Thus, the interagency working group sought to establish a sensible framework that effectively protected human health and the environment while providing breathing room for a burgeoning industry. Scientists also wanted the freedom and flexibility to engage in research and did not want Congress to pass unduly restrictive laws (Mandel 2006).

The U.S. Federal government set forth its policy statement on the regulation of agricultural biotechnology in a document entitled the Coordinated Framework for Regulation of Biotechnology (OSTP 1986). This publication in the Federal Register established the regulatory roles for Executive Branch agencies in ensuring the safety of biotechnology research and products for human health and the environment, and in addressing a previous policy proposal promulgated in 1984 with the same title (OSTP 1984). A 2 year public comment period helped to shape this policy statement in its evolution to the final 1986 publication. The working group formed under the OSTP concluded that the existing statutes and administrative agencies would be adequate for the regulation of biotechnology as long as they were under a common framework (Stepp 1999). The Coordinated Framework for the Regulation of Biotechnology set forth policy directing the oversight of biotechnology under EPA, USDA, FDA, NIH, the National Science Foundation (NSF), and the Occupational Safety and Health Administration (OSHA) dependent on the type of genetic modification under development. The Framework also established a Biotechnology Science Coordinating Committee to ensure timely and coordinated regulatory decision making, interagency communication, discuss jurisdiction over products of biotechnology, and to keep track of the changing scene in biotechnology (Stepp 1999).

The Coordinated Framework was guided by several principles, including the concept of a case-by-case review of new products, assessing the risk associated with the product and not the process itself, and that genetically engineered organisms do not differ fundamentally from their non-GE counterparts (i.e., the same parameters of biochemistry, genetics and physiology apply to all organisms regardless of origin). It was further anticipated that the technology would evolve and regulations, as well as administrative procedures, would also need to evolve to adapt to novel products of biotechnology (OSTP 1986). It was noted early on, however, that both pesticidal and non-pesticidal microorganisms would require further regulatory refinement as compared to other organisms known at the time the Framework was released to the public.

As a result of existing statutory mandates and regulatory history, three agencies were selected to oversee the primary regulation of agricultural biotechnology: the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS), the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA). With each agency regulating products of biotechnology under separate statutory mandates, the individual product may be regulated by more than one agency (Table 1.1). It is important to remember that each agency will view the product differently based upon their statutory responsibilities. These regulatory triggers will be explained in the sections below dealing with individual agency oversight. While not discussed herein, States may also regulate these products under their laws beyond the realm of Federal mandates.

The first approved environmental release of a GE organism occurred in 1987 following the approval by the National Institutes of Health (NIH) and the U.S. Environmental Protection Agency (EPA). This first release was a field test of "ice-minus" bacteria used for preventing frost damage on strawberries (Marchant 1988). These were strains of *Pseudomonas syringae* and *Erwinia herbicola* with mutations in a gene encoding an ice-nucleation protein that is normally expressed on the bacterial cell surface, but not in "ice-minus" strains. This approval sparked a heated controversy, including several court cases, challenging the NIH decision and questioning the ability of federal agencies to address hazards to ecosystems in light of the uncertainties (Wrubel et al. 1997). Although this ice-minus phenotype is outside the normal scope of EPA oversight related to pesticides, the controversy erupting publically when the test was first proposed in 1984 and the lack of an established regulatory framework for GE organisms at that time led to EPA becoming the default agency for oversight (Bill Schneider, EPA, personal communication, 2011).

This chapter and many others in this book deal with the primary statutes which grant authority to Federal agencies for oversight of biotech products. It is at least worth mentioning that many other statutes may play a role in regulation of biotech products in specific instances at both the Federal and State levels. For example, the National Environmental Policy Act is significant in the regulatory process at USDA-APHIS and FDA (Belson 2000; Mandel 2006). The Endangered Species Act is also considered as part of the risk assessment process for USDA-APHIS, EPA and FDA when making environmental risk management decisions. Additionally, individual

Table 1.1 Oversight of genetically engineered plants and traits in the US

Trait phenotype/crop	Agency	Statutory authority[a]
Disease/insect resistance in food or feed crop	USDA-APHIS EPA/US FDA[b]	Plant Protection Act – plant pests, weeds and environmental effects FIFRA/FFDCA – PIP pesticides; environmental, food and feed safety FFDCA – food and feed safety
Herbicide tolerance in food or feed crop	USDA-APHIS[c] EPA[d] FDA	Plant Protection Act – plant pests, weeds and environmental effects FIFRA/FFDCA – herbicide use on crop; environmental effects, food and feed safety of herbicide residues FFDCA – food and animal feed safety
Herbicide tolerance in ornamental/ non-food crop	USDA-APHIS EPA	Plant Protection Act – plant pests, weeds and environmental effects FIFRA – herbicide use on crop, environmental effects
Quality enhancement traits for food or feed crop	USDA-APHIS FDA	Plant Protection Act – plant pests, weeds and environmental effects FFDCA – food and feed safety
Flower color enhancement in a non-food crop	USDA-APHIS	Plant Protection Act – plant pests, weeds and environmental effects

[a]Primary statutory authority, however, other statutes may apply under certain circumstances. It should be noted that all agencies involved are subject to the provisions of the Endangered Species Act
[b]FDA oversight may be voluntary consultation when trait is not a food additive
[c]PPA requires an assessment of the GE crop to act as a plant pest as defined in 7CFR Part 340
[d]EPA does not regulate the HT crop plant, only the use of the herbicide, and its residues on the crop and potential non-target effects from the use of herbicide in a cropping situation

states may require more restrictive regulations for biotech products as they deem fit (Beachy et al. 1996). A further discussion of these statutes influencing oversight can be found in the OSTP archived biotech case studies (OSTP 2001a).

1.3 Coordinated Federal Framework

Biotech crops undergo a food safety and environmental review process conducted by the FDA, the EPA, and the USDA-APHIS. Each agency operates under their respective laws and regulations with some statutory overlap. The three agencies routinely interact while regulating GE organisms and make an effort to keep each other apprised of regulatory findings and decisions. Additionally, the OSTP oversees the Agricultural Biotechnology Working Group (ABWG), consisting of members from the regulatory agencies as well as several other Executive branch agencies. The purpose of the ABWG meetings is to ensure coordination among the U.S.

Federal government, and to provide a forum for open and free exchange of ideas, relative to the policy, regulation and use of biotech derived products in agriculture.

Briefly, each agency's roles are as follows[1]:

- *The USDA-APHIS protects agriculture and the environment from pests, diseases, and weeds.*
- *The EPA protects human health and the environment, using the standard of no unreasonable adverse effects upon man and the environment, as it evaluates plant-incorporated protectants, microbial pesticides, and intergeneric microorganisms.*
- *The FDA protects the safety of the food and feed supply.*

1.3.1 Role of the Animal and Plant Health Inspection Service

USDA-APHIS is responsible for protecting the United States' animal and plant resources from agricultural pests and diseases. Under the authority of the Plant Protection Act (June 20, 2000), APHIS regulations (7 CFR 340) provide procedures for obtaining a permit or for submitting a notification, prior to "introducing" a regulated article in the United States. A genetically engineered organism is considered a regulated article if the donor organism, recipient organism, vector or vector agent used in engineering the organism belongs to one of the taxonomic groups listed in the regulation and is also a plant pest, or if there is a reason to believe it is a plant pest (USDA-APHIS 2001).

The act of introduction includes any movement into (import) or through (interstate) the United States, or release into the environment outside an area of physical confinement. The regulations also provide for petitions for the determination of nonregulated status. Once a determination of nonregulated status is granted, the product (and its offspring) no longer requires APHIS review for movement or release in the United States. Transgenic plants that have been genetically engineered to express insecticidal proteins are considered regulated articles by APHIS unless and until they are granted non-regulated status through the petition process.

Unlike regulatory licensing as practiced under FIFRA by EPA, once GE organisms successfully complete a deregulation process, they are no longer subject to oversight by USDA-APHIS (Mandel 2006), although they may still be regulated under FIFRA if they are PIPs. Deregulated GE plants become nonregulated and are not required to submit yearly reports on sales or distribution to USDA-APHIS as they would be required to submit to EPA if they were registered as a PIP.

APHIS regulations part 7 CFR 340.6 (c)(4) describe the types of data and information that a developer must submit in support of a petition for nonregulated status.

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[1] http://www.aphis.usda.gov/publications/biotechnology/content/printable_version/BRS_CoordFrameBro.pdf

In part, these specifically include under a description of "known and potential differences from the unmodified recipient organism that would substantiate that the regulated article is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived," including effects of the regulated article on non-target organisms and indirect plant pest effects on other agricultural products and, under 7 CFR 340.6 (c) (5), data reports from field trials conducted under APHIS permit or notification that shall include "methods of observation, resulting data, and analysis regarding all deleterious effects on plants, nontarget organisms, or the environment."

Since the PPA relies on the determination of plant pest or noxious weed status as a trigger to regulation of GE organisms, and plant pests are defined rather broadly therein as essentially any organism causing harm to a plant or plant parts (Belson 2000), even the use of a plant pest (e.g., *Agrobacterium tumefaciens*) or a plant pest sequence (e.g., CaMV 35S promoter) as part of the transformation process may deem the resultant product a regulated article and under the oversight of USDA-APHIS. Interestingly, some plants engineered for herbicide tolerance while attaining the status of a noxious weed were not ultimately regulated under PPA as they were found to lack any plant pest sequences and no plant pest organism was used in their construction (USDA-APHIS 2011a, b).

1.3.2 Role of the U.S. Environmental Protection Agency

The EPA regulates pesticides under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). This Act requires that all pesticides sold or distributed in the United States must be registered with the EPA unless they are specifically exempted. The EPA also regulates the amount of pesticide residue that can be in or on the specific agricultural commodity the food or feed supply under the Federal Food, Drug, and Cosmetic Act (FFDCA). Most of this law is the purview of FDA, but the pesticidal authority to establish tolerances or exemptions from the requirement of a tolerance rests with EPA. FDA does maintain enforcement authority under FFDCA in cases where an illegal pesticide residue persists on a food or feed product.

EPA also regulates intergeneric microorganisms, under the Toxic Substances Control Act (TSCA) section 5, that are not covered by other statutes and are manufactured, imported, or processed for commercial purposes. Agricultural purposes can include biofertilizers (e.g., nitrogen fixers, mycorrhizae, phosphate solubilizers, etc.), algal biofuels, pesticidal intermediates, and perhaps, biosensors. Under the Coordinated Framework, EPA promulgated regulations for intergeneric microorganisms under TSCA which were finalized in 1997 (see Chap. 4 for greater detail).

Regulations for Biotechnology Notification prior to small scale field testing of engineered microbial pesticides were finalized in 1994 and existing regulations for PIPs were finalized in 2001. Chapter 4 in this volume contains further details on FIFRA and TSCA regulation of microbes and Chap. 10 details the regulatory requirements for PIPs under FIFRA and FFDCA.

Microbial pesticides can be naturally occurring or genetically engineered. Genetically engineered microorganisms are regulated using the same data requirements used for naturally occurring microbial pesticides (See 40 CFR part 158.740). Additional information may be required concerning the genetic engineering process used and the results from that process, however, the toxicity and pathogenicity evaluation is identical to that used to assess the non-GE counterpart MPCA. EPA requires a Biotechnology Notification be issued prior to small scale field testing of genetically engineered microorganisms at any size of environmental release to allow EPA to determine if an Experimental Use Permit is needed (See 40 CFR part 172 subpart C). When testing 10 A or more terrestrially or 1 A aquatically, EPA requires an Experimental Use Permit before field testing naturally occurring or genetically engineered microorganisms when used as microbial pest control agents (MPCA). Under FIFRA, microbial biotech products, as with all other pesticides, must be evaluated for their risks and benefits. Before any registration is granted, OPP considers such issues as potential adverse effects to non-target organisms, environmental fate of the microorganism, and the potential pathogenicity and infectivity of the microorganism to humans and other animals.

PIPs are defined as pesticidal substances "intended to be produced and used in a living plant, or in the produce thereof, and the genetic material necessary for the production of such a pesticidal substance". The PIP also includes any inert ingredient contained in the plant, or produce thereof (40 CFR 174.3). Inert ingredients may include herbicide tolerance traits and antibiotic resistance markers when they are used in the development of a PIP product. PIPs are regulated under FIFRA as pesticides and require a tolerance exemption or exemption from the requirement of a tolerance under FFDCA when the PIP is expressed within a food or feed crop (Table 1.1). The genetic material necessary for the production of such a pesticidal substance also meets the FIFRA statutory definition of a pesticide because such genetic material is introduced into the plant with the intent of ultimately producing a pesticidal effect even though the genetic material may not, itself, directly affect pests. Both the insecticidal protein and its genetic material are regulated by EPA; the plant itself is not regulated by EPA. This is a key distinction between PIP regulation by EPA and regulation of GE crops by USDA-APHIS and FDA.

EPA also issues experimental use permits (EUPs) for field trials of PIPs that are more than 10 acres cumulative area across the United States when targeting a single pest or pest complex.. These EUPs are intended to serve as a mechanism to collect field data in support of an eventual Section 3 registration. The 10 acre cutoff for regulatory oversight is based upon the concept that a small acreage results in a small overall environmental exposure and is, therefore, not likely to result in an adverse effect upon the environment. It should also be noted that in most instances, the USDA-APHIS is regulating the field trials at any size area under a permitting system. However, if the PIP could be in the food or feed supply or be fed to animals which would enter the food supply at less than 10 A area of field testing, then a tolerance or tolerance exemption must be obtained before field trials are performed regardless of whether an EUP is required or not. A company may choose to test several closely related transformation events under one EUP, but a commercial

registration would only be for a PIP resulting from a single transformation event (EPA 2011a).

PIPs are pesticides and are therefore regulated under FIFRA. Under FIFRA Section 3, EPA registers PIPs to be sold and distributed with the consequent regulations under 40 CFR. EPA evaluates each PIP application to determine whether its proposed use would cause unreasonable adverse effects on man and the environment. In order to avoid potential unreasonable adverse effects, the Agency may impose (and has imposed) conditions on registration of PIPs (e.g., conditions to slow or eliminate insect resistance; EPA 2001a). When the PIP expressing plant may enter the food or feed stream, FFDCA section 408 is also applicable to the PIP crops or human food or animal feed products derived from them.

Under FFDCA, EPA establishes tolerances, or in the case of the PIPs registered to date, tolerance exemptions, wherein no numerical maximum level or quantity of the pesticidal substance residue is denoted. Such exemptions from the requirement of a tolerance are based upon the absence of adverse toxicological outcomes during acute toxicity testing. EPA evaluates each PIP application to determine whether dietary exposure to the residue of any PIP in food or feed is safe, i.e., whether that there is a reasonable certainty of no harm resulting from aggregate exposure to the pesticide, which includes all anticipated dietary exposures and all other exposures for which there is reliable information. The tolerance exemptions issued allow PIPs to be used in foods with a reasonable certainty of no harm. Due to the ubiquitous nature of nucleic acids in food and feed, and the lack of demonstrable toxicity from their consumption, all nucleic acids as present within PIPs are exempted from the requirement of a tolerance under FFDCA.

Based on laboratory studies, field trials, and other information, EPA scientists assess a wide variety of potential effects associated with the PIP. These areas will be discussed in Chaps. 10, 11 and 12 according to scientific discipline.

EPA considers public comments for PIP regulatory actions and often holds FIFRA-proscribed Scientific Advisory Panel meetings charging outside experts to peer review EPA's risk assessments, when EPA identifies specific scientific questions or concerns that need additional consideration (EPA 2011b). All public comments are reviewed for their potential impact upon decision making (i.e., risk management) and responded to publically. Time frames and fees for EPA pesticide registration decisions vary based on the type of action. EPA uses a fee-for-service system associated with its pesticide registrations and experimental use permits under the Pesticide Registration Improvement Act (PRIA) of 2004, as amended. Under PRIA, an applicant pays a fee according to the specifics of the regulatory action sought (e.g., EUP, registration, tolerance) and receives a definitive timeline for decision making. Fees vary by action and portions of the fee may be waived depending on the affiliation of the applicant; researchers associated with a government agency will have all fees waived, those from universities or small companies may have a portion waived and those from large companies (i.e., >500 employees) will generally not receive a waiver. This fee for service approach is in contrast to FDA and USDA-APHIS who do not charge fees for reviews or consultations regarding GE microbes or plants.

1.3.3 Role of the Food and Drug Administration

The Food and Drug Administration uses the food safety and food additive authorities in the Federal Food, Drug and Cosmetic Act of 1938 (FFDCA), as amended, to regulate the safety of biotech foods. Under these laws, FDA operates a voluntary premarket notification and consultation system that provides biotech companies an opportunity to demonstrate that foods produced from their biotech crop are as safe as their traditional counterparts.

If biotech food contains a protein or other new substance that is not "generally recognized as safe" (GRAS), the food must go through a formal FDA premarket approval process in which the sponsor must prove scientifically that the new substance in the food is safe. Note that the new substance does not include pesticides, which are regulated by EPA, but rather something like a modified oil profile or a protein altered such that it is no longer an allergen.

FDA's oversight of biotech foods is managed through the Division of Biotechnology and GRAS Notice Review, Office of Food Additive Safety, in FDA's Center for Food Safety and Applied Nutrition (CFSAN), which coordinates reviews with FDA's Center for Veterinary Medicine (CVM). CFSAN regulates GE crop plants which are not PIPs and contain food additives (FDA 1997, 2005a), whereas CVM regulates GE animals containing new animal drugs (OSTP 2001b).

In 1992, the FDA issued a policy statement regarding how the agency intended to regulate human foods and animals feeds derived from new plant varieties, including varieties developed using DNA technology, which were referred to as "bioengineered foods." In general, the FDA announced that bioengineered foods would be regulated no differently than foods developed through traditional plant breeding. As a class, bioengineered foods did not require special labeling nor were they subject to premarket approval. The FDA looks to the objective characteristics of the food and its intended use, not the method by which the food was developed.

The FDA also acknowledged the food industry's long-standing practice of consulting with the FDA in the early stages of developing food through new technologies. This practice, although not required, allows the agency to identify and address issues regarding foods and food ingredients before they are marketed. The FDA expressed its expectation that such consultation would continue with regard to bioengineered foods. In 1997, the FDA issued guidance on procedures for these consultations (FDA 1997).

A company that intends to commercialize a bioengineered food meets with the FDA at an "initial consultation" to identify and discuss possible issues regarding safety, nutritional, or other regulatory issues. A "final consultation" is held once the company believes it has developed the data and information necessary to address issues or concerns raised by the FDA.

The FDA consultation process does not constitute a formal review, as would occur with a food additive for example, but rather it is a voluntary consultation. During this iterative process, the FDA Center for Food Safety and Applied Nutrition performs a comparative assessment of the composition of the GE crop and its non-GE

counterpart (FDA 1997). In instances where the proximate analysis and the examination of allergens and anti-feedants suggests that there is no significant difference between the GE and non-GE counterparts, the FDA indicates that it has no further questions regarding the use of this food or feed product in commerce, but it remains the responsibility of the manufacturer to ensure the safety of the food or feed product (Belson 2000). This finding by the FDA, while made on a voluntary basis, indicates that the GE food or feed product is 'as safe as' its non-GE counterpart. The agency does not deem a GE food or feed crop as 'safe' per se. To date, all GE food and feed products have undergone a consultation with FDA CFSAN prior to marketing even though the process is voluntary. The Flavr Savr™tomato was the first commercialized GE food crop and the only one to date to undergo formal review by FDA as a food additive, at the request or insistence of the developer Calgene (FDA 2005b). This review considered the presence of the neomycin phosphotransferase enzyme in the food product as this enzyme was used as a selectable antibiotic resistance marker in the development of the product.

1.4 Trends

The U.S. regulatory system has matured over the last 30 years by remaining adaptable and flexible as well as by being responsive to input from stakeholders. Following advances in molecular biology and rDNA techniques in the 1970s, genetic engineering of microbes, then plants, soon followed. As with most new technologies, a level of uncertainty led to apprehension among scientists and the general public once applications of biotechnology were becoming a reality (Pizzuli 1984; Griffin 1988). The Asilomar Conference in 1975 served to address some of these concerns although not all attendees were in agreement on how products of biotechnology should be regulated and by whom (Howland 1987; Marchant 1988; Barinaga 2000).

Following the establishment of the Coordinated Framework for Regulation of Biotechology in 1986, the role of the three principal regulatory agencies was somewhat clearer, however, the three agencies needed to further develop policies and practices. This was only the beginning. Guidance documents promulgated by regulatory agencies started to take shape, but these are an ongoing process to this day as they continue to respond to advances in biotechnology.

One of the authors recalls that in the late 1980s and early 1990s, even simple experiments with recombinant plasmids performed in debilitated laboratory strains of *E. coli* (e.g., K-12) triggered a laboratory inspection by both the USDA-APHIS and local university Institutional Biosafety Committee representative. Adherence to the NIG Guidelines (NIH 1976) was agreed to laboratory access limited in terms of public invitations for tours. Following applications to USDA-APHIS to receive strains of *Agrobacterium* to be used in transformation protocols, laboratory and growth room facilities were inspected and later audited to ensure all GE materials were kept confined under lock and key and uninvited personnel could not gain access to these tissue cultures bacterial stabs! Instructions were also given to placard

the doors and refrigerators with biohazard insignias. Transgenic cotton plants were not allowed in the university greenhouse, but had to be keep in a locked storage room outfitted with high intensity lamps! Early measures were rather cautious to say the least. We have come a long way since 1987.

References

Barinaga M (2000) Asilomar revisited: lessons for today? Science 287:1584–1585

Beachy R et al (1996) Appropriate oversight for plants with inherited traits for resistance to pests: a scientific review of a policy proposed by the U.S. Environmental Protection Agency, Plant-pesticides subject to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA); American Institute of Biological Sciences, Coordinating society, Institute of Food Technologists, Chicago, IL, USA

Belson NA (2000) US regulation of agricultural biotechnology: an overview. AgBioForum 3:268–280. http://www.agbioforum.orghttp://www.agbioforum.org. Accessed 21 Dec 2011

EPA (1994) PR EPA proposes regs to address plant-pesticides. http://yosemite.epa.gov/opa/admpress.nsf/618671a0682e1f4d85257359003d4802/9521c6b8cecbcc3c8525646d0077b428!OpenDocument htm. Accessed 21 Dec 2011

EPA (2001a) Biopesticides Registration Action Document – *Bacillus thuringiensis* plant-incorporated protectants, D. Insect resistance management. http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad. Accessed 21 Dec 2011

EPA (2001b) Questions & answers – biotechnology: final plant-pesticide/plant-incorporated protectant (PIPs) rules. 19 July 2001. http://www.epa.gov/scipoly/biotech/pubs/qanda.pdf. Accessed 21 Dec 2011

EPA (2011a) Current & previously registered section 3 PIP registrations. http://www.epa.gov/opp00001/biopesticides/pips/pip_list htm. Accessed 30 Nov 2011

EPA (2011b) Scientific advisory panel, A to Z subject index. http://www.epa.gov/scipoly/sap/tools/atozindex/atozindex htm. Accessed 21 Nov 2011

ERS (2011) Adoption of genetically engineered crops in the U.S.: extent of adoption. US Department of Agriculture, Economic Research Service. http://www.ers.usda.gov/Data/BiotechCrops/adoption htm. Accessed 21 Dec 2011

FDA (1997) Consultation procedures under FDA's 1992 statement of policy – foods derived from new plant varieties. Office of Premarket Approval, Center for Food Safety and Applied Nutrition and Office of Surveillance and Compliance, Center for Veterinary Medicine, Food and Drug Administration http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/Biotechnology/ucm096126.htm. Accessed 10 Jan 2012

FDA (2005) Guidance for industry: pre-petition consultations for food additives and color additives. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/ucm055270.htm. Accessed 21 Nov 2011

FDA (2005b) Bioengineered Foods Statement of Robert E. Brackett, Ph.D. Director Center for Food Safety and Applied Nutrition Before the Senate Committee on Agriculture, Nutrition and Forestry 14, June 2005. http://www.fda.gov/newsevents/testimony/ucm112927.htm. Accessed 21 Nov 2011

Griffin K (1988) Public fears factored into gene-altered bacteria tests. Los Angeles Times, 18 April 1988. http://articles.latimes.com/1988-04-18/local/me-992_1_altered-bacteria. Accessed 29 Dec 2011

Howland HJ (1987) Regulation for the deliberate release of biotechnology products. Ohio J Sci 87:174–181

ISAAA (2012) International Service for the Acquisition of Agri-biotech Applications, biotech country facts and trends. http://www.isaaa.org/resources/publications/biotech_country_facts_and_trends/default.asp. Accessed 06 Jan 2012

Jepson P (2003) The philosophical perplexities and ethical enigmas of biotechnology: an examination of the regulatory process in the United States. In: Eaglesham A, Ristow S, Hardy RWF (eds) Biotechnology: science and society at a crossroad, NABC report 15, pp 197–202, Ithaca, NY

Mandel GN (2006) Toward rational regulation of genetically modified food. Santa Clara J Intl Law 4:21–59. http://www.scujil.org/sites/default/files/volumes/v4_MandelArticle.pdf. Accessed 21 Dec 2011

Marchant GE (1988) Modified rules for modified bugs: balancing safety and efficiency in the regulation of deliberate release of genetically engineered microorganisms. Harvard J Law Technol 1:163–208

NIH (1976) NIH Recombinant DNA research guidelines July 7, 1976 Fed Reg 41:27902

OSTP (1984) Office of Science and Technology Policy, Proposal for a coordinated framework for regulation of biotechnology, 49 Fed Reg 50856 (1984)

OSTP (1986) Coordinated framework for regulation of biotechnology. 51 FR 23302–93, 26 June 1986

OSTP (2001a) CEQ and OSTP assessment: case studies of environmental regulations for biotechnology. http://www.whitehouse.gov/administration/eop/ostp/library/archives. Accessed 12 Jan 2012

OSTP (2001b) Case study No. 1: growth-enhanced salmon. http://www.whitehouse.gov/files/documents/ostp/Issues/ceq_ostp_study2.pdf. Accessed 21 Nov 2011

Patterson LE, Josling T (2001) Regulating biotechnology: comparing EU and US approaches. Originally presented at the Western Economic Association International 76th annual conference, San Francisco, 8 July 2001. http://aei.pitt.edu/28/1/TransatlanticBiotech.pdf. Accessed 21 Dec 2011

Pizzuli E (1984) Foundation on economic Trends v. Heckler: genetic engineering and NEPA's EIS requirement. Pace Env Law Rev 2:138–165 http://digitalcommons.pace.edu/pelr/vol2/iss1/7. Accessed 01 July 2011

Schneider W (2011) Personal communication, U.S. Environmental Protection Agency, Office of Pesticide Programs (retired), 15 November 2011

Stepp DL (1999) The history of FDA regulation of biotechnology in the twentieth century. Food Drug Law 46:1–116. http://leda.law.harvard.edu/leda/data/257/Stepp,_David_00.pdf. Accessed 21 Dec 2011

USDA-APHIS (2011a) News release: USDA responds to regulation requests regarding Kentucky Bluegrass, 01 July 2011. http://www.aphis.usda.gov/newsroom/2011/07/pdf/KY_bluegrass.pdf. Accessed 21 Dec 2011

USDA-APHIS (2011b) Review of petition to add genetically engineered Glyphosate-tolerant Kentucky bluegrass to the federal noxious weed regulations, 30 June 2011 Emergency and Domestic Programs Kaplan, DT. http://www.aphis.usda.gov/plant_health/plant_pest_info/weeds/downloads/Kentucky-BG/KY-BG-FNWPetitionReview.pdf. Accessed 21 Dec 2011

Wrubel RP, Krimsky S, Anderson MD (1997) Regulatory oversight of genetically engineered microorganisms: has regulation inhibited innovation? Environ Manage 21:571–586

Chapter 2
Regulation of Genetically Modified Crops in USA and Canada: Canadian Overview

Stuart J. Smyth and Alan McHughen

Abstract This chapter provides a non-technical review of the regulations pertaining to GM crops in Canada. It provides a detailed overview of the science-based regulatory framework that exists to regulate biotechnology and hence, genetically modified crops. Canada utilizes a three-pronged regulatory approach that differentiates between agriculture (crops), food and the environment. We discuss the development of the regulatory frameworks pertaining to biotechnology derived crops and also provide a present day review of these frameworks.

Keywords Biotechnology • Genetically modified organisms • Plants with novel traits • Regulation

2.1 The Canadian Regulatory Framework

Canadian regulators established a new classification of plants to deal with the potential risks that had a probability of developing following the application of new genetic technologies to the science of plant breeding. The development of Canadian regulations for the initial innovative crops were based on science and subsequent regulatory changes have continued to be based (mainly)[1] on science. In accordance

[1] It should be noted that both Health Canada and Environment Canada use non-scientific rDNA processes as regulatory triggers.

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with recommendations from various international scientific societies, the regulations focus on the end product, not the process used to create the product. To this end, Canada developed regulations for plants with novel traits (PNTs). Plants that are classified as PNTs are plants that do not have a history of production and safe consumption in Canada. They may have been introduced from elsewhere, or genetically modified using genetic engineering, mutagenesis, or any other breeding method (CFIA 2004a).

However, differing from the US regulatory system, some rDNA developed plants are not PNTs, which creates some confusion for crop developers. Most jurisdictions trigger regulatory scrutiny for every new rDNA insertion into a plant cell's genome, but the CFIA triggers regulatory scrutiny only when a plant expresses a new trait, whether or not the plant is a product of rDNA. Plants developed using traditional breeding, not rDNA, have triggered regulatory review for expressing novel traits. An example is a conventionally bred barley variety expressing low phytate from the University of Saskatchewan. A plant developed using rDNA but not expressing a novel (or, in this case, unapproved) trait would be exempt, even if it is a new or different transformation or insertion event. For example, if a transgenic PNT were assessed and approved, then a subsequent plant of the same species transformed with the same DNA construct and expressing the same traits as the approved variety, any cultivars derived from this new 'event' would not trigger regulatory scrutiny as a PNT, as it would not be novel. However, the developer would still have to fulfill variety registration requirements prior to commercial release, and does retain the obligation to report any subsequent unusual or unexpected observations.

2.1.1 Overview of the Regulatory Framework

The regulation of products created via biotechnology is the responsibility of several federal government agencies: the CFIA, Environment Canada (EC) and Health Canada (Table 2.1). Using legislation from four different Acts, the CFIA is responsible for plants, animal feeds, fertilizers and veterinary biologics. The Office of Plant Biosafety was established within the CFIA to co-ordinate the safety evaluation of novel foods. Environment Canada acts as a regulatory safety net for products of biotechnology, where they have the regulatory mandate for all animate products of biotechnology for uses not covered under other federal legislation. Environment Canada regulates biotechnology within the scope of the Canadian Environmental Protection Act (1999). Through the Food and Drugs Act, Health Canada oversees the regulation of foods, drugs, cosmetics, medical devises and pest control products. All safety assessments are conducted based upon scientific principles developed through expert international consultations with the World Health Organization (WHO), Food and Agriculture Organization (FAO) and the Organization for Economic Co-operation and Development (OECD) (Harrison 2001).

Table 2.1 Legislation governing biotechnology in Canada

Agency	Product	Act
CFIA	Plants with novel traits	Seeds Act
	Novel fertilizers and supplements	Fertilizers Act
	Novel livestock feeds	Feeds Act
	Veterinary biologics	Health of Animals Act
Environment Canada	All animate products of biotechnology for uses not covered under other federal legislation	Canadian Environmental Protection Act (1999)
Health Canada	Novel foods	Food and Drug Act
	Pest control products	Pest Control Products Act

Source: CFIA (2005a)

All novel trait products, prior to receiving registration approval, are thoroughly tested by the CFIA, Environment Canada and Health Canada officials using scientific approaches. Officials from all departments work together on a new variety application. Officials do not conduct or redo the scientific experiments and research information that is submitted by the applicant (usually a private company or public university); rather, they analyze the data submitted and may redo portions of the experimentation to corroborate results. Frequently, government officials will ask the applicant to provide them with additional information regarding specific segments of the application, which may result in the applicant conducting additional scientific experiments. Upon the review of all information, the variety is accepted if all conditions are fully met and rejected if any condition is not deemed to be acceptable.

The following section will first focus on the scientific/governance aspect of developing these new regulations and then second, on the process used to develop the regulations. This section will identify the risks that the regulatory framework strove to address and the subsequent section will discuss the regulatory development process, identifying the objectives of the framework and the collaboration involved in the development process.

2.2 The Regulation of New Crop Varieties

The traditional governance system for crop agriculture is based on an extensive horizontally-based public/private regulatory system (Smyth et al. 2004). Risks are managed by various stakeholders depending on the stage of the variety development. Breeders, whether private and public, are responsible for managing any risks in their research programs as long as the materials remain in contained conditions (e.g. in laboratories or under glass). Once the breeder has developed a cultivar that shows agronomic or other merit, is genetically unique and stable it is ready to be examined for registration and the formal system takes over.

In the production system, the public sector has tended to establish the general environment for private actors to effect transactions. The Food and Drugs Act (1985) set rules for human consumption,[2] the Feeds Act (1983) sets maximum tolerances of nutrients for livestock feed, the Seeds Act (1985) specifies the performance standards for new germplasm and the Canadian Grain Commission sets and monitors the standards for the seeds trade.

These three Acts are designed to establish standards for risks related to plant agriculture. The main quality attributes of the Seeds Act are genetic uniformity, stability and uniqueness ('uniqueness' here means the new cultivar must be genetically modified or genetically different from all earlier varieties). However, this Act also establishes thresholds for environmental safety risk aspects such as geneflow, invasiveness, weediness and impact on non-target organisms. The Feeds Act defines the thresholds for the potential risks due to allergenicity, toxicity, digestibility and dietary exposure relating to animal feeding. The Food and Drugs Act establishes risk thresholds for allergenicity, toxicity, metabolization, nutrition and dietary exposure relating to human consumption. The integration of these three Acts into the regulatory framework for new plant varieties is designed to identify potential risk categories and ensure that any new plant variety is benchmarked to existing varieties already determined to be safe for human and animal consumption.

The Seeds Act is the first point of quality assurance, as new varieties must on average at least equal the quality (in set parameters) of contemporary commercial varieties. With some exceptions, all new varieties of grain and oilseeds generally flow through the same system in Canada, with higher levels of oversight for those that involve novel traits (novel traits are described below in greater detail). The variety registration system stipulates that any new variety developed within 30 major agricultural crops has to receive variety approval prior to importing of seed, advertisement for sale or sale of seed. This process is in place to ensure that the new variety being submitted for approval exceeds or is at least equal to existing varieties. This is done to ensure that the overall quality of the crop is constantly improving, a manifestation of the 'merit system'.

When the crop breeder develops the new cultivar, they begin initial field trials to provide the evidence to evaluate the environmental risks of the new cultivar and to assess its agronomic merit (e.g. yield, disease resistance, time to maturity, quality and any other traits). After several years in these preliminary trials, the best performing lines are transferred to nationally coordinated "Co-op" trials for large scale evaluations in wide geographical distribution, in competition with other candidate cultivars from other breeders of the same crop. Most new cultivars require 3 years of these national Co-op field trials to gather sufficient data to support commercial release. However it is possible to take only 2 years in certain cases.

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[2] Health Canada sets policies and standards for food safety. However, if a modified plant will be used as animal feed and has the potential to introduce harm to humans when the animal is consumed as food, it is the Canadian Food Inspection Agency that enforces this aspect of the policies and standards.

Once the field trial data is gathered it is submitted to the relevant official variety recommending committee. At this point, the public/private aspect of variety registration comes fully into effect as the merit assessment of new variety applications is conducted by official recommending committees. There are 21 recommending committees, recognized by the CFIA. These committees are comprised of academic, government and industry representatives; they evaluate the agronomic, grain quality and disease rating data, make the performance comparisons and then these variety experts make a decision as to whether the merits of this particular cultivar meet (or exceed) the quality standards for that particular variety. If the decision is in favour of the new cultivar then the breeder receives notification that the committee supports the registration and commercialization of the new variety. When the new cultivar has been evaluated and supported for registration by an appropriate recommending body of experts, a dossier is submitted to the Variety Registration Office (VRO) within the CFIA. The VRO reviews the submission data and has the authority to request additional information from the breeder prior to granting variety approval. The VRO, acting for the Minister of Agriculture, retains final authority to grant variety approval in Canada.

There are four variety approval options when making approval decisions (CFIA 2000). The typical option is to grant national approval to the new variety, meaning that there are no restrictions on sale of seed or production of crop anywhere within Canada. Regional registration can be granted to ensure that a crop variety is only produced within a defined geographic area. The geographical separation between the western prairie crop area and the lower Ontario, Quebec and Maritime growing area often defines the regional approval. Contract registration is granted to varieties that are required to be segregated from other like varieties for crop safety reasons (see Smyth and Phillips 2002 for more details on segregation systems). Finally, interim registration can be approved that establishes a fixed duration for the variety approval.

Once a variety is approved, the Canadian Seed Growers Association manages the seed multiplication system, specifying the tolerances for off type seeds and substandard materials, and the retail seed business, by overseeing the sale of seeds by registered name. After harvest, the Canadian Grain Commission takes over quality assurance for much of the product, setting and enforcing grades and standards for the trade. Within this context, spot markets have relatively efficiently managed the commercialization of a large number of new varieties over the years (Kennett et al. 1998).

This public/private governance framework minimizes the biosafety and commercial risks associated with the approval of new crop varieties. The role of the various recommending committees is essential as it ensures that experts working with that specific crop type are the ones that make the initial recommendation regarding approval. This recommendation is not made by arms-length bureaucratic scientists, but by a group of individuals with appropriate expertise and hands-on experience. This integration into the regulatory framework has resulted in a variety approval system that consistently works towards improved safety and quality and thus, risk minimization.

2.3 The Development of the Initial Framework: 1988–1995

2.3.1 The Scientific Approach to the Initial Genetically Modified Crop Regulations

By 1986, transformed plants with new transgenic traits were available and ready for field testing in Canada. The science of transgenic plants was well in advance of the governance capacity as there was no regulatory protocol in place at this time. The number of field trials was relatively small and those conducted in 1986 and 1987 followed the protocol used for all previous field trials with new crop varieties. By the spring of 1988, federal permits were required to plant a field trial with a transgenic plant variety. Following the initial permits, the governance process was conducted by the use of periodic directives issued by federal regulators. These directives were issued following considerable contact and discussion with the industry stakeholders.

The trials were conducted to gather the data required by the Seeds Act, Feeds Act and the Food and Drugs Act as described above. The regulators also needed evidence on the characterization of the transformation system, the nature of the carrier DNA, genetic material delivered to the plant, the components of the vector and a summary of all genetic components. In addition, the regulator required an array of data to assess the inheritance and stability of the genetic modification (e.g. Mendelian segregation) and a description of the novel traits (e.g. Southern analysis and qualitative ELISA analysis of the gene expression levels).[3]

Once confined field trials were authorized, they were undertaken following a strict set of guidelines and standards, which, while national in application, were drawn from international evidence of the appropriate risk management procedures and the latest international biosafety evidence. While the regulators were responsible for auditing and enforcing the rules on trials, these trials were usually managed directly by the research firm or by a contractor (in Canada, the various research farms operated by various universities or Agriculture Canada have managed many of the trials under contract with the companies).

By 1992, the breeders conducting field trials had gathered enough data to demonstrate intergenerational stability, agronomic efficacy and commercial promise and began to develop their regulatory package of evidence to present to the regulators to assess the safety of the products. In Canada, this required extensive data on the toxicity of the novel gene products (e.g. a series of toxicity studies with animals and non-target species). The product proponents also had to provide scientific studies on the nutritional aspects of the novel trait and plant for both humans and livestock and comparisons of the amino acid sequences of the novel trait to known allergen proteins. Finally, the proponents were required to provide a package of studies on the

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[3] See www.inspection.gc.ca/english/plaveg/bio/subs/subexe.shtm for a detailed list of what this involves.

environmental impact of the novel traits on soil, weeds, wild relatives and non-target organisms. McHughen (2000) published a photographic histogram of the volume of data required to satisfy regulators of the health and safety of transgenic crops (in his case a transgenic flax variety) – the pile of studies and reports exceeded 3 ft. for the transgenic product, versus a typical example of about 30 pages for a conventionally-bred variety.

The results of the field trials, food, feed and environmental reviews were then examined by the appropriate regulators. In Canada, Health Canada (HC) undertook the food safety review while the environmental and animal health reviews were conducted by forerunner agencies of the CFIA.[4] In each case they had enabling standards embedded in legislation or regulation which needed to be made specific for each product or technology. That process involved extensive negotiation between the regulator and the product proponent, supplemented with reference by the regulator to experts in other national regulatory systems and to those outside the regulatory system.

Finally, the initial GM crop varieties (three new trait canola varieties and one flax variety) were assessed by a committee of researchers operating under the authority of the Seeds Act – they analyzed the candidate varieties against standard, commercially grown 'check' varieties – and then the committee recommended registration to allow them commercial release for sale to farmers. The USA and some other countries do not have this regulatory step. At that point a blended public-private quality control system took over.

For the initial GM canola varieties this was administered by the Western Canadian Canola Rapeseed Recommendation Committee (WCCRRC). This committee is comprised of over 30 individuals representing public and private breeders, the canola crushing industry, the Canola Council of Canada and the larger canola industry. The WCCRRC evaluate new varieties against the 'check' varieties and recommend varieties for release. This standard has been backstopped by the Canola Council of Canada trademark on canola, which specifies that products must have less than 2% erucic acid and 30 μmol of glucosinolates per 100 g of dried meal. Furthermore, the new variety approval system periodically raises the bar for new varieties by choosing a new 'check' variety as the base, which incrementally raises oil and meal properties, grain yields and disease resistance standards to slowly but continually improve the overall quality of the crop over time.

The regulatory approval process for the canola varieties was completed in February 1995 when the Pest Management Regulatory Agency submitted recommendations for approval for two varieties of GM canola to the Expert Committee for Canola. Agriculture and Agri-Food Canada approved the two varieties for unconfined release in March 1995, meaning that large scale commercial production of GM crops could occur in Canada.

While the scientific risks were resolved by the close involvement with the academic community and industry in developing a regulatory directive on the biology

[4] These agencies were all departments within the Department of Agriculture, now the Department of Agriculture and Agri-Food Canada.

of the species to establish familiarity, this did not address all the risks. Canada was not alone in having to develop a regulatory framework for transgenic crops as the US and Europe were heavily engaged in this as well. Many of the breeders involved at this time recognized that new regulations were inevitable and that close collaboration with the regulators would be advantageous. While there were scientific risks that needed to be addressed through regulation, Canada also had to develop this regulatory framework to remain competitive at an international level. The actions of the US and Europe meant that if Canada commercialized transgenic products without a thorough regulatory framework, the perceived lack of safety regulation could be viewed as a concern, thus legitimizing the denial of market access to Canadian products. To ensure that trade barriers did not arise, all recognized that a thorough risk analysis and approval system would be an essential component of advancing the industry of transgenic plants.

The important observation from the development of regulations for these first generation GM crops is that the regulators were openly accepting of industry and academic stakeholder involvement. At the time of commercialization, the mid-1990s, the regulators operated from the perspective that once the scientific risks were satisfactorily addressed, the technology was allowed to proceed unimpeded by regulatory interference.

2.3.2 The Process of Developing PNT Regulations in Canada

This section discusses the interaction between science and governance that occurred as the regulatory framework was developed in Canada.

The initial workshop to address the regulatory framework that would be required for the successful commercialization of transgenic crop technologies was organized in 1988 by the Canadian Agricultural Research Council (CARC). There were 108 attendees for this workshop, 65 from the various government agencies and research organizations, 27 from numerous private industry firms, 14 from Canadian universities and two guests from the USDA.

The objectives of the workshop focused on an assessment of the status of agricultural biotechnology in Canada. The first objective was to engage in a current assessment of the regulatory environment for agricultural biotechnology products in Canada. The second objective was to identify how this Canadian situation compared to those of the US and Europe. The final objective was to define what regulatory concerns existed at this point in time from the perspective of the industry and the regulators.

This workshop produced the following key recommendations designed to improve the regulatory process and which provided the basis for the development of the PNT regulatory framework:

- those plants which possess characteristics or traits sufficiently different from the same or similar species should require an assessment of risk;

- the product, not the process should be regulated; and
- the categories of novel herbicide tolerance, novel pesticidal properties, novel stress tolerances and novel compositional changes were raised as categories of concern. (CARC 1988)

Over the next 3 years, the Director of the Animal and Plant Health Directorate within the Food Production and Inspection Branch of Agriculture Canada, would convene periodic *ad hoc* meetings of varying representation to discuss pertinent issues. It was not until 1992 that a formalized structure was put in place to deal with the regulatory changes that would be required. It was decided in April 1992, that a standing advisory committee would be established with the following mandates:

- provide information and guidance on the regulation of plant biotechnology;
- assist in the development of a consistent regulatory approach; and
- assess and evaluate regulatory requirements for field testing and commercialization of genetically modified plant material. (Agriculture Canada 1992)

The Plant Biotechnology Advisory Committee would have formalized representation as well and the membership consisted of representatives from 11 various agriculture related societies and associations.[5]

In 1992 the Food Production and Inspection Branch contracted with Dr. Wally Beversdorf (Chair of the Department of Crop Science at the University of Guelph) to develop draft protocol and assessment criteria for unconfined release of PNTs. This initiative, when taken in consideration with a series of workshops held across Canada between January and March 1993, produced a draft set of regulations. A workshop was held November 8–10, 1993 in Ottawa to discuss the draft regulations. The draft regulations were shared with attendees prior to the workshop.

The workshop consisted of numerous presentations from a variety of stakeholders that had been invited to participate in the workshop. The objectives of the workshop were: building consensus on the approach to regulate PNTs; consistency with existing regulations; sharing of information; and developing working relationships (CFIA undated).

The principles of the federal regulatory framework were identified as: building on existing legislation and institutions; upholding health and environmental safety standards; harmonizing with national priorities and standards; using risk-based assessments and methodologies; assessing products, not processes; and developing a favourable climate for investment, development and innovation by adopting sustainable products and processes.

Much discussion was given to the concept of substantial equivalence for products derived by biotechnology, especially the difference between 'familiarity' and 'substantial equivalence'. Familiarity was described as an extensive knowledge of

[5] Membership consisted of: Canadian Seed Growers Association, Canadian Seed Trade Association, Crop Protection Institute, Genetics Society of Canada, Canadian Society of Agronomy, Confederation of Canadian Faculties of Agriculture and Veterinary Medicine, Plant Biotechnology Institute, Expert Committee on Weeds, Canadian Society on Botany, Canadian Phytopathological Society and the National Seed Potato Bureau.

factors relating to the production of a particular crop species that allows for decisions pertaining to safety to be made, whereas substantial equivalence would apply to those new crop types whose safety could not be identified from a standard risk assessment.

Unfortunately, because there is no standard accepted definition of 'substantial equivalence', and agencies use differing definitions, a firm decision was not made by Canadian regulators pertaining to the use of substantial equivalence in regulatory actions. This inconsistent definition contributed to the ongoing confusion over use of the term. This confusion was witnessed in the report of the Royal Society (The Royal Society of Canada 2001). Canada has a *de facto* application of substantial equivalence in that regulators apply regulations to the resulting product, not the process used to create the product, which is contradictory to the wording in CFIA regulations.

The CFIA states that '…a plant with a novel trait is one that is not "substantially equivalent" to existing plants of the same species cultivated in Canada…' (CFIA 2005b p.1), but this is incorrect: as the progeny of approved PNTs are not considered novel. The Royal Society report was widely criticized in the scientific community, partly because it assumes *a priori* that transgenic plants are suspect and so, they suggest, scientific evidence must be presented to prove them safe. This is faulty on two points: first, there is no scientific reason to suppose plants developed using rDNA are any more risky than plants developed using other technologies; and second, science cannot prove something safe. Health Canada, on the other hand, states that '…substantial equivalence is not to be used as a decision threshold and GM-products should be subject to a rigorous scientific assessment of their potential for causing harm…' (Health Canada 2001 p.1). In fact, Health Canada goes on to identify that substantial equivalence is not uniformly applied in federal regulations. Ultimately, while substantial equivalence for PNTs was not defined within the developing regulations, some form of it has been practiced by the regulators.

Key recommendations for the draft regulations were that time should not be a factor in approving these new technologies, rather safety should be the chief concern and safety should be established regardless of the time taken to do this. International acceptance of the products was identified as crucial for commercial success for these new crop technologies. Participants acknowledged that there are risks, but the focus must be given to identifiable, science-based risk, not hypothetical socio-economic risks. The final recommendation dealt with the importance for regulatory harmonization within North America. Harmonization would ensure that the ultimate regulatory framework would not hinder the competitiveness of this emerging industry in Canada (CFIA undated).

In March 1994, a follow-up workshop was convened by the Plant Products Division (PPD) of Agriculture Canada with the objective of reviewing the regulations that had been redrafted following the November 1993 workshop. The PPD wanted an expert review of specific guidelines that had been developed to provide for the unconfined release of new canola and flax varieties prior to the regulations being released (Agriculture Canada 1994). The PPD called the members of the Plant Biotechnology Advisory Committee (see footnote 5 for the list of members) together to provide their insights. While this workshop focused on unconfined release for

canola and flax, the group also held initial discussions regarding unconfined release for maize, soybeans and potatoes. The feedback from the committee members was incorporated into the document released for public comment and the biotechnology industry believed they had cleared the final regulatory hurdle.

In June 1994, those involved in the development of the regulatory framework were surprised and alarmed when the Feed Section of the Plant Products Division informed participants of the Plant Biotechnology Advisory Committee that they would initiate the development of their own regulatory guidelines for the use of genetically modified plant material in livestock feed. The Feed Section sought participation by experts to form the Transgenic Plants as Livestock Feed Advisory Committee. Membership of this committee consisted of 16 experts from various involvements in biotechnology.[6] A workshop was held in Ottawa from September 21–22, 1994, and draft guidelines were developed and sent out for public comment on November 22, 1994. Twenty five comments were received and were incorporated into a revised draft of the regulations that was sent back to the members for comment. Comments were due back from committee members by March 15. It is interesting to note that the first decision document approving unconfined release of a PNT crop (AgrEvo's ammonium-tolerant canola) was approved on March 13, 1995, as this was 2 days prior to the end of the above comment period and well in advance of the final approval of regulations developed by the Feed Section. The second was given to Monsanto's Roundup™ herbicide-tolerant canola on March 24, 1995.

The 7 year process of developing the regulations for PNT crops was time consuming, yet the process was scientifically justified and successful as there have been no documented problems resulting from the 15 years of commercial PNT crop production in Canada. The scientific risks and the governance aspect of risk management were captured within the PNT regulatory framework. However, the process was not without challenges as was identified by the lack of any defining characteristics regarding substantial equivalence. The regulatory mandate was to a certain degree unfocused, as was evidenced by the late involvement of the Feed Section. Regulatory integration of the Feed Section needed to occur in harmony with the larger actions of the PPD. The result of this scattered approach to developing PNT regulations was that the commercial release of PNTs in Canada was delayed by 1 year.

2.4 Canada's Present Regulatory Framework

2.4.1 The Canadian Food Inspection Agency (CFIA)

In Canada, all commercialized GM plants to date have been considered to contain novel traits and, therefore, have been assessed for safety. However, the approach used by the CFIA does not mean that all PNTs are developed through genetic

[6] Environment Canada was sporadically engaged in the regulatory process at this time.

modification. Novel traits can be developed through various techniques (other than genetic modification) such as mutagenesis, somaclonal variation and other forms of what in other countries are considered 'traditional' breeding. Canada does not use the breeding process as a trigger for regulation, but instead focuses on the features of the product. For example, the non rDNA somaclonal variant Clearfield canola was considered a PNT and regulated as such, but Normandy flax, which was also bred using somaclonal variation, was not.

Because of this, government evaluators carefully assess potential impacts before these modified plants can be released into the environment. Environmental safety assessments examine five broad categories of possible impacts of a PNT. These are:

- the potential of the plant to become a weed or to be invasive of natural habitats;
- the potential for gene flow to wild relatives;
- the potential for a plant to become a plant pest;
- the potential impact of a plant or its gene products on non-target species; and
- the potential impact on biodiversity (CFIA 2004b).

Due to the above definition and the subsequent assessment categories, every herbicide tolerant (HT) variety application that the CFIA receives, is treated as a PNT, regardless of the technology used to create the HT variety. While there are very few crop varieties approved with stacked traits (maize, cotton and potato), a HT variety that has additional traits stacked with it, such as drought tolerance, would be given consideration for variety approval under the following CFIA directives:

- Directive 94–08: Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits.
- Directive 95–03: Guidelines for the Assessment of Novel Feeds: Plant Sources.
- Directive D-96-13: Import Permit Requirements for Plants with Novel Traits, and their Products.
- Directive 2000–07: Guidelines for the Environmental Release of Plants with Novel Traits within Confined Field Trials in Canada.

Using these directives, the CFIA assesses all PNT variety applications for environmental release and use as animal feed. It is no longer possible to get split approval for a crop variety in Canada where it would be approved for use as animal feed but not human consumption.

There are three stages in the assessment process for a PNT variety. In Stage 1 of the development of a new PNT variety that is intended for unconfined environmental release and/or use as a livestock feed, the plants are required to be grown in a contained facility (i.e. greenhouse or laboratory growth chamber). Growing conditions in these types of facilities follow biosafety guidelines that have been established by Health Canada and the Medical Research Council. Research institutions may develop and require that codes of practice be followed in addition to the above.

In Stage 2, the PNT variety developer must submit an application to the CFIA and receive authorization to conduct confined field trials in Canada. Directive 2000–07 is used to establish how many trials are allowed in Canada, the size of the plot and isolation distances that are required. The CFIA notifies each province where

field trials applications have been received for and provincial authorities are given a 30 day comment period. The field trials are conducted over several years in various locations that represent potential adoption regions and the data that is produced by these trials are used to provide information to the CFIA for the safety assessments in Stage 3.

Stage 3 is designed to address the five priority categories listed above. To provide the necessary information to satisfy these questions, the product developer is required to submit scientific data that has been gathered from the field trials. The CFIA has a database of scientific studies that it will draw upon to review the studies and may commission additional studies if required. Peer reviewed journal articles are also utilized as sources of relevant information. The scientific data that is required for the CFIA to undertake the safety assessment includes: identification and classification of the PNT; modification methods; description of the novel trait(s); environmental data; livestock feed data that is comprised of nutritional, toxicity and allergenicity data (CFIA 2006).

It is at this stage where the bottleneck in the system exists. The lack of a data 'roadmap' that could inform breeders as to the specifics of what is required is becoming a barrier to commercialization. As the science of genetic modification continues to advance, increasingly more knowledge about GM plants is available. This increase in knowledge about GM plants does not change the probabilities of a risk event, but it does change the regulatory perceptions of a risk event. As the science of genetic modification advances so too should the regulation of the products but the regulatory advances have to be based on quantifiable increases in risk probabilities. This is not the case with the regulatory creep that is occurring in Canada.

Following the review of the scientific data a decision document is drafted and sent to the product developer as well as posted on the Internet. This document explains how the review took place and provides a basis for the final decision that was rendered. If at any point following this, additional scientific information becomes available regarding the crop variety, the product developer is required to report this information to the CFIA who will undertake a re-evaluation based on the information. At this point, the CFIA regulation process is complete and the product developer is eligible to apply to the CFIA for unconfined commercial production of the new PNT crop variety.

The requests for additional scientific data are coming too late in the regulatory process and this results in commercialization delays which prevents producers from having the opportunity to adopt improved crop varieties. Smyth and Phillips (2001) estimated that in the case of GM canola, a 1-year delay in commercialization would have cost the entire canola industry C$100 million.

2.4.2 Health Canada (HC)

Unlike the CFIA, which uses a product trigger, Health Canada defines novel food as foods resulting from a process not previously used for food, products that do not

have a history of safe use as a food or foods that have been modified by genetic manipulation, genetically engineered foods or biotechnology-derived foods (Health Canada 2006a). Health Canada assesses the safety of all GM and other novel foods proposed for sale in Canada. Companies are required to submit detailed scientific data for review and approval by Health Canada, before such foods can be sold or as animal feed if the modified feed has the potential to introduce harmful components into the portion of the animal being consumed as food.

Health Canada is also responsible for the environmental assessment of products regulated under the Food and Drugs Act, including novel foods. This activity is required by the New Substance Notification Regulations of the Canadian Environmental Protection Act (1999). Two branches within Health Canada, the Health Products and Food Branch (HPFB) and the Healthy Environment and Consumer Safety Branch (HECSB) started working in 2001 to develop new regulations to assess the impact on the environment and on human health of new substances used in these products. This process was known as Health Canada's Environmental Impact Initiative (Health Canada 2006b).

Health Canada does not review all foods new to the Canadian market but only those that are deemed novel. Therefore, the concept of prior safe use as a food was introduced to exclude foods new to the Canadian market which have a history of safe food use in other countries, from being the target of a novel food notification. Secondly, the concept of 'major change' was introduced into the novel food definition in order to avoid the potential of a minor processing change to trigger a novel food notification. This approach intended to restrict novel food notifications due to introduction of new processes only to those that are truly new and cause substantial changes in the composition of the food.

A major change with respect to a food is defined as a change peripheral to the manufacturer's experience or generally accepted nutritional or food science theory. This would place the modified food outside the accepted limits of natural variations for that food with regard to:

- the composition, structure or nutritional quality of the food or its generally recognized physiological effects;
- the manner in which the food is metabolized in the body; or
- the microbiological safety, the chemical safety or the safe use of the food. (Health Canada 2006c)

The challenge of this approach is that the transparency regarding the required submission of scientific data for regulatory approval is even less transparent that the CFIA's process. The less precedence there is of use of a novel food product, the less transparency.

Regulators at HC take the data from the field trials conducted by the product developer that relate to the category for novel foods. This is when the nutritional, toxicity and allergenicity data is reviewed and assessed. Additional data is needed to satisfy risk assessments regarding dietary exposure, metabolization and micro-biological safety. One salient feature of the HC regulatory process is HC's use of experience from other jurisdictions. If a PNT product has a history of safe production

and consumption in another country, then this history is admissible as data for regulatory approval in Canada. Health Canada is unique among the PNT regulatory bodies in this as the CFIA and EC will not allow a history of safe production and consumption elsewhere as admissible evidence.

Health Canada has established criteria for the assessment of novel foods that provide information to establish the safety of the novel food. Written notification is required at least 45 days prior to the sale or advertising for sale of any novel food. Health Canada is required to respond within 45 days of receipt of the notification regarding its acceptability for sale. If additional information is required to properly establish the safety of the product, such information will be requested in writing and "the clock" is stopped, thus extending the period. The applicant is not permitted to sell or advertise the product until the additional information requirement is fulfilled and the Department has agreed to the acceptability of the product.

Once the Novel Foods Section of HC receives the application for a new PNT food product from the product developer, there are four reviews required. The product developer has to address environmental safety, chemical safety, nutritional changes/stability and microbial hazards.

Once the scientific review of data is complete, HC can request additional information, which then requires another scientific review of the new data. If there are no requests at this point, a draft ruling is developed by the Novel Foods Section that then goes up the bureaucratic ladder for review. Senior management within HC has the right to request additional information from the product developer at this stage and this process would trigger another scientific review. If the drafted proposal is acceptable, then a letter is sent to the product developer informing them of this and the Decision Document is posted on the Internet. At this point, the product developer may safely market the PNT product or crop variety.

Again, it is the requests for additional information that acts as a commercialization barrier. The risk spectrum would appear to be limitless when dealing with novel foods and this greatly frustrates breeders. Many of the scientific advances in detection of food risks now allow for testing to be done at previously undetectable levels. This raises the cost of regulatory approval as breeders have to conduct additional research to be able to quantify the new risk detection levels. This would not be an issue for breeders (or certainly less of a one) if there were peer review articles in existence that quantified the need for greater risk detection levels. Unfortunately, these articles do not exist and the increased regulatory scrutiny would appear to be not risk-based.

2.4.3 Environment Canada (EC)

Environment Canada regulates products of biotechnology using The New Substances Notification Regulations (NSNR) of the Canadian Environmental Protection Act (1999) (CEPA). Environment Canada uses this legislation to anticipate and prevent the introduction of new substances that may pose unacceptable risks to human health and the environment. The NSNR is a federal initiative designed to respond to

concerns over recent growth in the diversity and quantity of commercially available substances. As part of a cradle to grave management approach to toxic substances, the provisions for substances new to Canada are intended to ensure that no new substance is introduced into the marketplace before an assessment of its toxicity has been completed. Toxicity, as defined in CEPA, refers to risk to human health or the environment (Environment Canada 2007). Features of the new substances program include criteria for identifying new substances, a mechanism for assessing new substances and, when necessary, the enabling powers to implement specific controls.

Substance is defined by CEPA as including animate matter, i.e. organisms (Environment Canada 2005). A 'new' substance is a substance that is not listed on the Domestic Substances List (DSL). The DSL is a compilation of substances that were in commerce between January 1, 1984 and December 31, 1986, according to the criteria set out in CEPA. An eligible organism is one that was in use in Canada between 1984 and 1986 such that its entry into the environment was unrestricted. The DSL is the sole standard against which a substance is judged to be 'new' to Canada. With few exceptions, any substances not on this list are considered new and EC must be notified prior to importation or manufacture. While there are 35 existing biotechnology substances listed on the DSL, products derived from the process of biotechnology are classified as 'new substances' under CEPA.

The assessment process is initiated when EC receives a new substance notification prepared by the product developer that proposes to import or manufacture a substance. New substance notifications must contain all required administrative and technical data and must be provided to EC prior to manufacture or import. Notification information is jointly assessed by the Departments of Environment and Health to determine the potential adverse effects of the substance on the environment and human health. This assessment, which must be completed within a time specified by the NSNR, will result in:

- a determination that the substance is not suspected of being toxic;
- a suspicion that the substance is toxic, which may require: (i) controls on, or prohibition of, import and manufacture, or (ii) prohibition pending submission and assessment of additional information determined to be required by the Departments; or
- limiting the purpose for which a substance may be used to permit the waiver of information requirements (Environment Canada 2005).

The regulations covering chemical or polymer substances have been in effect since July 1, 1994, while those covering biotechnology substances including organisms, or products of micro-organisms have been in effect since September 1, 1997.

New substances that require regulation under the DSL are divided between Environment Canada and Health Canada. Health Canada reviews the scientific data that relates to human exposure and potential human toxicity risks. Environment Canada reviews the scientific data that relates to non-human risks. Following a review of the data, officials from both departments meet to determine the level of risk assessment, which can result in three potential courses of action. The first is that the new substance is deemed to be safe which requires no further action to be taken

by regulators. The second outcome is that a level of risk has been identified and the new substance is placed on the list of priority substances. The final outcome is that the new substance is identified as a toxic substance (e.g. PCBs) and placed on the toxic substances list, which means that the substance is to be eliminated and prevented from entering the environment.

Of the three regulatory agencies, EC is the least engaged with the PNT process. The additional scientific data requests during the regulatory approval process by the CFIA and HC contribute to a lack of transparency and certainty regarding the regulatory approval process. The absence of the ability of variety developers to know how much scientific research is sufficient to complete the dossier frustrates them. Plus, the regulators' demands for additional scientific data takes time and costs money to compile. The lack of transparency as to what data is sufficient contributes to the delay of the innovation process within crop agriculture. However, there have also been concerns expressed by developers of microbial biotechnology regarding EC's assessment process, it has limited application to the issues related to the PNT regulatory process.

The three-pronged regulatory approach used by Canadian regulators, is consistent with the regulatory approach taken in the US. While the nomenclature differs slightly, the regulatory mandates of the three American regulatory bodies is very comparable to those of the three Canadian regulatory bodies. Regulatory harmonization efforts were first initiated in 1998 and continue to be held on a regular basis. The result of this dialogue process is the mutual recognition of molecular characterization data by regulatory agencies in both countries (CFIA 2001) While this can be a frustratingly slow process, progress is being made. While the American and Canadian regulatory frameworks are not flawless, they are an international beacon for science-based regulation.

2.5 Conclusions

The Canadian PNT regulatory system for innovative crop based technologies is founded in science and has proven efficient in ensuring that any risks have been minimized. The investment that was spent in drafting the regulations over a decade ago has provided a return many times over as the adoption rates of GM canola, maize and soybeans has been very high, thereby providing benefits to Canadian producers with no documented damage to health or environment. While this process was necessary to commercialize the initial GM varieties, the time has come to revisit these regulations.

The rigors of the regulatory requirements in terms of the cost of conducting the studies necessary to gather sufficient data to meet the demands of the regulators for aspects such as gene flow, allergenicity and toxicity are pushing public researchers out of the variety development industry. Public research institutions have limited budgets and simply do not have the finances to undertake the expensive research required to satisfy regulators. The concern within the seed development industry is that the

commercialization of new traits will only be done by large multinational seed developer, thereby having a potentially large negative impact on continuing development of crop varieties that are best situated for Canada, such as canola and flax.

There is justified concern about the increase in regulatory requirements for GM crop varieties as this increase in regulation is not justified by any increase in risk. The correlation between innovative GM or PNT crop varieties and increased risks to human safety have not been scientifically documented. As the scientific capability to detect an increasing number of potential risk factors increases, Canadian regulators are in some ways, acting like a sponge by simply increasing all of the regulatory requirements without relinquishing any risk factors that have been consistently addressed through over 20 years of research and commercial use. At some point, the regulatory system will have to decide which risk factors can be efficiently addressed as the process of trying to address each and every existing and new risk factor will stretch the regulatory capacity far beyond economical efficiency, resulting in costly commercialization delays.

Greater understanding of GM crops types exists following 15 years of production. Unfortunately, this has not facilitated improvements in the regulatory approval process for GM crops. While there has been consistency in the decision making process, the continued use of case-by-case[7] assessments has resulted in the situation where there is no identifiable regulatory template for developers to follow. This has created the scenario where no seed developer submitting an application package for regulatory approval of a new PNT knows what scientific data is required, or how much data is enough. At present, breeders submit a volume of data that they perceive to be enough for approval according to the application form, but fully expect the regulators to request additional information. The problem that breeders have with this process is that they have no idea of how much data is enough nor a final decision timeline as the data request process is open-ended and supplying additional data can frequently result in further additional demands from regulators.

While the regulatory framework in Canada is imperfect, it is preferable to the situation in most other nations. The fact that regulations are scientifically based and apply to the product, not the process, has resulted in a risk efficient regulatory framework. Improvements are required, and in this sense, the regulatory framework for GM plants is no different than the regulatory framework for any other industry; increased regulation slows the innovative process.

When the regulatory frameworks in Canada were being developed, the detailed level of regulation was, in part, justified because of the lack of familiarity that existed within the research community regarding rDNA technology. The regulatory community believed that a rigorous regulatory framework was required in the initial period of GM crops to ensure that risks were addressed and that the products were safe. During the meetings and workshops used to develop the frameworks in both countries, it was envisioned that the level of rigorous regulatory oversight would

[7] Case-by-case is based on the trait that has been inserted and this means the risks may vary and therefore, so does the regulatory focus.

only exist until a degree of familiarity was reached. There has now been 15 years of commercial production in North America and if one includes the early field trials, nearly 25 years of experience, knowledge and information has been generated. In the minds of many, the degree of familiarity regarding rDNA breeding techniques has been reached, yet the relaxing of regulations remains unseen. Surely, the time has come to engage in a rigorous review of existing regulatory requirements, to relax or remove outdated requirements and to encourage further innovations in plant breeding.

References

Agriculture Canada (1992) Letter of invitation to participate. Mimeograph

Agriculture Canada (1994) Plant biotechnology advisory committee: summary of proceedings. Queen's Printer, Ottawa

Canadian Agricultural Research Council (1988) Proceedings of the workshop on regulation of agricultural products of biotechnology, Ottawa

Canadian Environmental Protection Act (1999) http://laws-lois.justice.gc.ca/eng/acts/C-15.31/. Accessed 09 Aug 2012

Canadian Food Inspection Agency (2000) Procedures for the registration of crop varieties in Canada. http://www.inspection.gc.ca/english/plaveg/variet/proced/regenr_promode.shtml#4

Canadian Food Inspection Agency (2001) Environmental characterization data for transgenic plants intended for unconfined release. http://www.inspection.gc.ca/english/plaveg/bio/usda/appenannex2e.shtml

Canadian Food Inspection Agency (2004a) Directive Dir2000-07: conducting confined research field trials of plants with novel traits in Canada. http://www.inspection.gc.ca/english/plaveg/bio/dir/dir0007e.shtml

Canadian Food Inspection Agency (2004b) Directive 94–08: assessment criteria for determining environmental safety of plants with novel traits. http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9408e.shtml

Canadian Food Inspection Agency (2005a) How are plants with novel traits assessed for their long term safety by the Canadian Food Inspection Agency (CFIA)? http://www.inspection.gc.ca/english/sci/biotech/reg/equive.shtml

Canadian Food Inspection Agency (2005b) Regulation of agricultural biotechnology in Canada. Queen's Printer, Ottawa

Canadian Food Inspection Agency (2006) Regulation of agricultural biotechnology in Canada an educator's resource. http://www.inspection.gc.ca/english/sci/biotech/gen/educ_postsece.shtml

Canadian Food Inspection Agency (Undated) Workshop on regulating agricultural products of biotechnology. www.inspection.gc.ca

Environment Canada (2005) A guide to understanding the Canadian Environmental Protection Act, 1999. http://www.ec.gc.ca/CEPARegistry/the_act/guide04/s5.cfm

Environment Canada (2007) Overview of CEPA 1999. http://www.ec.gc.ca/CEPARegistry/gene_info/Overview.cfm

Harrison B (2001) Regulating novel foods in Canada. Presentation to the conference the convergence of global regulatory affairs: its potential impact on international trade and public perception. Saskatoon, Canada, 19–20 Mar

Health Canada (2001) Action plan of the Government of Canada. http://www.hc-sc.gc.ca/sr-sr/pubs/gmf-agm/substan_table_e.html

Health Canada (2006a) Genetically Modified (GM) foods and other novel foods. http://www.hc-sc.gc.ca/fn-an/gmf-agm/index_e.html

Health Canada (2006b) Environmental impact initiative. http://www.hc-sc.gc.ca/ewh-semt/contaminants/person/impact/about-impact/index_e.html

Health Canada (2006c) Guidelines for the safety assessment of novel foods. http://www.hc-sc.gc.ca/fn-an/legislation/guide-ld/nf-an/guidelines-lignesdirectrices_e.html

Kennett J, Fulton M, Molder P, Brooks H (1998) Supply chain management: the case of a UK baker preserving the identity of Canadian milling wheat. Supply Chain Manage 3:157–166

McHughen A (2000) Pandora's picnic basket: the potential and hazards of genetically modified foods. Oxford University Press, New York

Smyth S, Phillips PWB (2001) Competitors co-operating: establishing a supply chain to manage genetically modified canola. Int Food Agribus Manage Rev 4:51–66

Smyth S, Phillips PWB (2002) Product differentiation alternatives: identity preservation, segregation and traceability. AgBioForum 5:30–42

Smyth S, Phillips PWB, Kerr WA, Khachatourians GG (2004) Regulating the liabilities of agricultural biotechnology. CABI Publishing, Wallingford

The Royal Society of Canada (2001) Elements of precaution: recommendations for the regulation of food biotechnology in Canada. http://www.rsc.ca//files/publications/expert_panels/foodbiotechnology/GMreportEN.pdf

Chapter 3
Regulation of Genetically Modified Crops in USA and Canada: American Overview

Alan McHughen and Stuart J. Smyth

Abstract This chapter provides a non-technical review of the regulations pertaining to GM crops in the US, a detailed overview of the science-based regulatory framework that exists to regulate biotechnology and, hence, genetically modified crops. The USA utilizes a three-pronged regulatory approach that differentiates between safety threats to agriculture (crops), food and the environment. We discuss the development of the regulatory frameworks pertaining to rDNA (biotechnology) derived crops and also provide a present day review of these frameworks.

Keywords Biotechnology • Genetically modified organisms • Genetically engineered organisms, biosafety, regulation, policy

3.1 Introduction

Over the last century, agriculture in general and plant breeding in particular has enjoyed vigorous research and rapid deployment of beneficial developments. Traditional forms of crop genetic improvements, such as selection and cross pollination, remain the standard tools in the breeders toolbox, but these have been supplemented with a range of new and specialized innovations, such as mutation

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breeding using ionizing radiation or mutagenic chemicals, wide crosses across species requiring human interventions such as embryo rescue, and rDNA mediated gene transfer, commonly called genetic modification (GM) or genetic engineering (GE).

The commercialization of genetically modified (GM) crops in the mid 1990s has witnessed the widest and most rapid adoption of all innovations in plant agriculture. In 2008, there were over 330 million acres of GM crops planted globally (James 2009), an increase of 22 million acres over 2009. Genetically modified crops were grown in 25 countries, 18 of which are considered developing countries. Ten countries in Latin America reported some level of GM crop production.

Numerous reviews document the various regulatory approaches employed by governments around the world to scrutinize risks associated with products of agricultural biotechnology. Many of these publications are scientifically sound, comprehensive and well documented, such as those emanating from the US National Academies of Science and other professional scientific organizations. But these are designed for a technically expert audience and regulatory professionals as casual but interested observers find them rough going. At the other extreme, a number of non-expert organizations publish reviews (mainly on the internet) claiming that Canada and the US give little or no regulatory scrutiny to genetically modified organisms (GMOs), also called transgenic or products of recombinant DNA (rDNA) technologies. While such reports are readily readable by non-experts, they are filled with errors and inaccuracies. In the middle are various newsletter type documents designed to give accurate and credible information to non-expert readers, but these typically focus on one agency or issue, insufficient to provide more than a superficial coverage of the field.

In this chapter, we present a review of the regulations pertaining to GM crops in the US, from a non-technical view. The chapter starts with a background to the development of the application of genetic modification to plant agriculture and then provides a review of the regulatory jurisdictions.

3.2 The Application of Genetic Modification to Plant Agriculture

In breeding a new crop cultivar, the breeder creates genetic variation (modification) and then identifies and 'selects' a new genotype with superior features. The selected genotype is then tested, maintained and nurtured through seed or vegetative propagation until sufficient stock is available for commercial release, presuming the ongoing testing provides satisfactory performance data. The breeder, in most cases, generates a population of plants with a uniform, identifiably novel, genetically stable genotype. The genetic variation may be generated by any of the methods mentioned above, or the breeder may carefully inspect and select among the natural genetic variation within any given population. Subsequent analysis and testing can take several years to ensure the beneficial features are indeed stable, heritable and expressed adequately over generations, with consideration taken of climatic fluctuations and across regional soil types. At the same time, the new genotype is

evaluated for agronomic (e.g. yield potential, reactions to relevant diseases, etc.) and product quality (e.g. oil profile for oilseeds, starch or flour for grains, etc.) characteristics. Finally, prudence (if not fear of liability and litigation) dictates responsible breeders evaluate the new genotype for any modulations in production of anti-nutritional components (for more information on general plant breeding procedures, see McHughen 2000).

Most crops produce undesirable substances such as allergens or toxicants, and years of breeding has successfully reduced – but not eliminated – these anti-nutritional substances. On rare occasion, new genotypes have expressed elevated levels of toxicants requiring rejection of otherwise good performing new cultivars. This plant breeding/selection process eliminates almost all potentially hazardous cultivars before farmers or consumers experience them, so this phenomenon is largely unknown by ordinary consumers. Also largely unknown to most consumers is the fact that virtually all foods contain small amounts of naturally occurring toxic substances which are harmless – or at least physiologically benign – when consumed in modest quantities (see, for example, Ames et al. 1990). On those rare occasions when natural toxins increase to potentially hazardous levels in commercially released cultivars, damage is limited by recognition of the problem and removal from the market. Probably the best known example is the Lenape potato, which had to be removed from the market after it was found to generate dangerously high levels of the toxic glycoalkaloid solanine (Akeley et al. 1968; Zitnak and Johnston 1970). Other examples of conventionally bred crops turning out to be unexpectedly hazardous are outlined by the US National Academy of Sciences (NAS) (NAS 2004a) and by Kuiper (2003).

The overall incidence of unexpected or unintentional genetic changes resulting in a hazardous crop – regardless of the method used to create the genetic modification – is extremely low as proven by the fact that there are so few documented examples (NAS 2004a). As a result of this traditional safety record, the US does not routinely regulate safety of new crop cultivars, relying instead on breeders and developers to exercise due diligence and prudence in their evaluations, a system that has worked remarkably well considering the lack of safety problems reported for new crop cultivars over the years, and continuing with newer methods of genetic modification as they are applied to crop improvement. For example, mutation breeding, using ionizing radiation or mutagenic chemicals to randomly disrupt DNA in crop plants has been used since the mid-twentieth century, producing over 2,200 registered crop cultivars (Maluszynski 1991; also see Food and Agriculture Organisation website at http://mvgs.iaea.org/) none of which have had the relevant DNA mutations fully characterized, and none of which have had to be removed from the market for safety reasons.

The application of mutagenesis to plant agriculture started initially with experiments in the 1920s. However, it was not until the 1950s that mutagenesis technology developed commercial value in relation to plant breeding. By the 1970s, mutagenesis breeding was widely accepted by plant breeders. It was also at this same time that a new technology was emerging, one based on a discovery from the 1950s by Watson and Crick: the double helix structure of DNA.

The historical assumption that changes in plants due to genetic modification in breeding are generally safe and benign was eventually challenged with the advent

of rDNA technology in the early 1970s. When Cohen and Boyer successfully connected two different pieces of DNA (Cohen et al. 1973) and thus initiated rDNA technology, the scientific community recognized not only the great potential for benefits of genetic modification, but also the potential for risk (Berg et al. 1974).

The 1973 Gordon Conference on Nucleic Acids was the first public event to call attention to potential risks of GM technology. The attending scientists '… were concerned that unfettered pursuit of this research might engender unforeseen and damaging consequences for human health and the Earth's ecosystems' (Berg and Singer 1995, p. 9011). As a result, in July 1974, a call for a public moratorium on any further rDNA research was issued to enable research scientists to learn more about the technology of gene splicing, including the safety of those working in the laboratories (*Ibid*.).

The Asilomar Conference, a multi-stakeholder event that brought together leading researchers and governmental regulators was subsequently held in 1975 to engage in a full and open discussion about the risks of genetic modification. The initial experiments involving rDNA research had raised many questions and concerns about liability and safety of the process. The conference focus was to discuss the risks of the research, the conditions needed to ensure that the risks were adequately addressed and such safety precautions as would be necessary to remove the moratorium and allow for future research to proceed safely. The striking aspect of this conference was that the world's leading experts on rDNA research developed the safety guidelines for subsequent research themselves, rather than having the guidelines developed and imposed on researchers by the government. The process was transparent and open to scientific and public scrutiny; it was in fact designed to reassure those concerned that appropriate steps were being taken to minimize any actual or hypothetical risks from being realized. As a result, interested and concerned scientists and others that attended the Asilomar Conference recommended a cautious evaluation of the rDNA technology and the products resulting from the use of rDNA, including genetically engineered organisms, (GEOs), more commonly called genetically modified organisms (GMOs) (Berg et al. 1975).

As researchers working with microorganisms and rDNA technologies gained a greater understanding of the technology, its application expanded to plants and animals. Presentations in 1983 at the Miami Winter Symposia on the molecular genetics of plants, and more fully documented in the scientific journals shortly after, witnessed Schell and van Montague describing transgenic tobacco resistant to methotrexate and kanamycin (Schell et al. 1983; Herrera-Estrella et al. 1983). Fraley, Rogers and Horsch from Monsanto detailed their success at generating transgenic petunia plants resistant to kanamycin (Fraley et al. 1983a, b) and Chilton talked about her team's work with inserting kanamycin resistance into tobacco (Barton et al. 1983).

The first commercial planting of a GM crop occurred in China in 1992 (James and Krattiger 1996). It involved the planting of 100 acres of transgenic tobacco for the purpose of seed multiplication. The first commercial production of a GM crop for food purposes occurred in 1994 in the United States by Calgene, with 10,000 acres of their transgenic, delayed-ripening tomato, Flavr Savr. By 1995, other crops were introduced, including cotton, canola, potatoes and maize. Research in this area now covers a wide range of GM varieties, from cereals and oilseeds to fruits and vegetables.

The commercial release of transgenic crops has created a split within the agricultural world, not only between countries, but within countries as well. Internationally, there has been a split between European Union (EU) member states and North America (Canada and the US). The EU views transgenic crops as a potential source of injury and some member states, such as Denmark and Germany, have introduced statutory liability regimes dealing with injury resulting from the production of GM crops (see Smyth et al. 2010, for greater detail on the Danish and German legislation). Nevertheless, Spain has produced between 45,000 and 55,000 acres of *Bacillus thuringiensis* (*Bt*) maize annually beginning in the late 1990s (Brookes 2002). Portugal and the Czech Republic also occupy a modest corner of GM maize production. Clearly, there are groups of producers within Europe that would adopt the technology of transgenic crops if they were allowed to do so without facing daunting market access restrictions (Smyth et al. 2004). The EU has disallowed domestic production of transgenic crops for large-scale food consumption, as well as the import of transgenic raw materials and unlabelled processed food products. Some genetically modified food products can be imported but must be approved in the EU and clearly labeled as GM products when the GM ingredient content is greater than 0.9 %. Otherwise, there is 'zero tolerance' for any GM content (McHughen and Wager 2010). Although the EU moratorium on GM crop production was supposed to have ended in April 2004, there has been a limited adoption of the technology in Europe.

By contrast, in North America, the production of transgenic crops and the consumption of the resulting food products have become the norm (Pew 2004). North America has approved the commercial release of a variety of transgenic food crops, which, by some estimates, are now incorporated into nearly 70 % of all processed foods. In 2009, 85 % of all the canola grown in Canada was transgenic (96 % of canola grown was herbicide tolerant as mutagenesis herbicide tolerant varieties accounted for the additional 11 %). The percentage for GM soybeans and GM maize in Canada was identical at 65 %. In the United States, the adoption rate for GM soybeans is 90 %, while the rates for cotton and maize range from 70 to 80 % (James 2009). The adoption of transgenic maize has increased rapidly with the growing market for ethanol production.

The adoption rate of GM crop technology is the most rapid of any crop technology in the history of crop agriculture. As with any innovation, the challenge that frequently arises is how to regulate the innovation (e.g. the Internet). The remainder of the chapter examines how the US government responded to this innovative technology as applied to agriculture.

3.3 The US Regulatory System

3.3.1 The Development of the Initial Framework: 1976–1989

Officials of the US National Institutes of Health (NIH) oversaw the development of containment standards for proposed rDNA research projects regarding viruses and bacteria that could potentially be harmful to humans if widespread exposure occurred. The recommended containment standards were largely suggestions and

voluntary, so the NIH formed a Recombinant DNA Advisory Committee (RAC) to mandate and establish as compulsory a set of rules regulating rDNA research in federally funded programs (NIH 1976; and later refined NIH 1978). This step was followed by similar compulsory mandates from the United States Department of Agriculture (USDA), the Environmental Protection Agency (EPA) and the Food and Drug Agency (FDA), thus effectively making rDNA research tightly regulated across the US, as virtually all rDNA research was conducted with either funding from or in association with one or more of these agencies.

When it became clear that crops improved using rDNA technologies were on the horizon, serious scientific regulatory analyses were initiated in the US, Canada and elsewhere, even before transgenic plants were first developed. Discussion of the potential for environmental or health risks associated with the application of rDNA technology to plants and crops was largely hypothetical at first. That limitation did not impede the scientific rigor of the potential hazards and fuel the demand for onerous regulatory scrutiny. The first such major report was issued by the Organisation for Economic Co-operation and Development (OECD) in 1982, just prior to the first reporting of transgenic plants. The OECD report was influential for a number of reasons, one being it standardized a definition of biotechnology as '…the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services' (OECD 1982). Shortly after, the US National Academy of Sciences issued a report on the risk assessment strategies in the US (NAS 1983). That was just in time, because later that year, the NIH authorized the first environmental release of a GMO (an ice-minus bacterium, described in Lindow and Panopoulos 1988) and the first transgenic plants were finally documented.

With technical, regulatory and even judicial developments (e.g. court challenges to the approval for environmental tests of the ice-minus bacteria) speeding up due to rapid technological developments and adaptation of technical advances from model species to commercially used species, the White House established a committee in the Office of Science and Technology Policy (OSTP) to recommend mechanisms to regulate the quickly advancing technology. The result was a publication outlining several important points. Most important, the OSTP concluded that rDNA was not inherently risky and that regulations should focus on the risks of products, not the processes used to develop them, so products of rDNA needed no new or special regulatory attention (OSTP 1986). Instead of creating a new regulatory structure, current legislation and regulations designed for current products would be adapted to deal with products of biotechnology. The coordinated framework also recognized the concept that GMOs are not inherently riskier than other, non-modified organisms or those genetically modified using other breeding methods. Finally, the OSTP document assigned regulatory priority among the relevant federal agencies: the USDA; the FDA; and the EPA (OSTP 1986).

Under the coordinated framework, the USDA would be the lead agency in evaluating plants as potential pests of agriculture, the FDA would review GMOs as potential threats to the food and feed supply, and the EPA would take priority in evaluating new GMOs with pesticidal properties (as well as those modified for novel pesticide usage). Most GMOs would be reviewed by two or even three agencies, depending

on the features. For example, a GMO where the resulting food or feed is not altered, or an ornamental crop variety with no intended food or feed use need not be reviewed by the FDA. However, every commercialized GMO plant to date has sought and completed a "voluntary" FDA consultation, even though the food or feed composition was identical to that of the non-modified comparator cultivars.

In recognizing the similarity of risks posed by products of novel biotechnologies with those of earlier breeding technologies, the coordinated framework rejected the need to create an entirely new bureaucracy to regulate the new products, as was recommended by some and instituted in, for example, the European Union (McHughen 2000). Instead, the US assigned risk assessment, analysis and management responsibility to those already holding and exercising appropriate expertise in existing agencies. Thus, for example, the regulatory expertise in pesticides within the EPA was tapped to regulate GMOs with pesticidal issues. Not only did this strategy obviate the cost of establishing a new layer of bureaucracy (to house new agencies to regulate GMOs exclusively), it also obviated the dilution of relevant expertise and resources caused by redistribution of those resources across different departments.

The OSTP regulatory approach was validated by the scientific community in a "White paper" from the National Academy of Sciences in 1987, reinforcing the concept that hazards resides in the product, not in the process by which it was made, and that rDNA posed no novel risks, in that the risks were 'the same in kind' as those presented by non-rDNA generated organisms (NAS 1987). A follow-up study considered more practical issues relating to a risk framework with environmental releases of GM microbes and plants (NAS 1989). Subsequent NAS scientific panels focusing on more and more specific aspects of biotechnology consistently came to the same conclusions. All methods of genetic modification, including traditional breeding, can give rise to potentially hazardous products, that biotechnology is no more likely to result in a hazardous product than traditional methods of breeding, and that the regulatory trigger for risk assessment should be based on the physical features of the product instead of the process by which the product was generated (NAS 2000, 2002, 2004a, 2010).

3.3.2 US Regulatory Agencies

3.3.2.1 The United States Department of Agriculture (USDA)

The USDA, through the office of Biotechnology Regulatory Services (BRS) of the Animal and Plant Health Inspection Service (APHIS), is primarily concerned with protecting agriculture and the environment (broadly interpreted) from potential pests (also broadly interpreted). The USDA regulates all GM plants prior to environmental release, including the import, interstate movement, small and large field trials and, of course, commercial (farm) cultivation. Initially, legislative authority was distributed among several statutes, including the Plant Quarantine Act, the Federal Plant Pest Act, and the Federal Noxious Weed Act, but was consolidated in 2000 in the federal Plant Protection Act (PPA).

Although the legal definition is complex, in simple practice, the USDA considers a 'regulated article' to be a plant and its progeny arising from a specific rDNA-transformed cell as an 'event'. For example, a maize plant, with a DNA construct carrying an inserted Bt gene would be a regulated article until such time (if ever) the USDA approves a petition for non-regulated status (see below). Another maize plant developed at the same time with the same gene construct, but regenerated from a different transformed cell, would be a different 'event'. The USDA justifies regulating each event separately because, it argues that the locus of insertion, which varies from one transformation event to another, even using identical DNA constructs and host plant genotypes, may give rise to different inserted gene expression patterns, gene product levels, and perhaps affect other features (e.g. via insertional knockout of endogenous genes) as well. Interestingly, once a 'regulated article' achieves 'non-regulated status', the GM plant can be released commercially with no further USDA regulatory oversight. Two such deregulated GM plants can even be bred together to produce a hybrid combining the novel features of each parent ("stacked"), without invoking additional USDA regulatory oversight.

However, this policy differs from that in other jurisdictions, notably the EU, where such stacked varieties are considered new and require separate regulatory assessment and approval. This difference may seem trivial, but will increasingly become problematic in future international trade.

Field Trials with GM Plants

The USDA initially authorized field trials under the Federal Plant Pest Act (FPPA) of 1957. This statutory authority was later consolidated and updated in the PPA of 2000.

In the 1987 *Federal Register*, the USDA published the first regulated procedure to allow field trials of GM plants (see 7 CFR 340). After the initial five applications in late 1987, (three were for herbicide tolerant tomato, two for herbicide tolerant tobacco; NAS 2000), field trial applications climbed dramatically. In the subsequent few years, the USDA issued 16 field trial permits in 1988, 30 in 1989, 51 in 1990 and 90 in 1991. To date, over 16,000 regulated field trials have been authorized. The GM plants included such species as tomato, tobacco, soybean, cotton, cucumber, poplar, potato, alfalfa, squash, walnut, melon, rice, canola, maize and others. Novel traits being tested included not only various marker genes, but agronomically interesting traits such as herbicide tolerance, insect protection, delayed ripening, nutritional enhancement and disease resistance.[1]

Notifications

Most field trials are approved under the notification procedure, which is the quickest and easiest process designed for the simplest or most familiar GM plants. Usually,

[1] The full listing of such USDA administered trials is available at: http://www.isb.vt.edu/cfdocs/fieldtests1.cfm.

notification involves submitting a letter to USDA's Biotechnology Regulatory Services (BRS) within APHIS documenting how the proposed GM plant meets six specified criteria and designated performance/characteristic standards. The criteria include such considerations as the GM plant not being of a noxious weed species and not transformed with human or animal pathogenic sequences. As well, the notification procedure does not apply to plant made pharmaceuticals (PMPs) or plant made industrial products (PMIPs). The notification can be used for field trial approval as well as importation and transport within the US of specified GM plants.[2]

Permits

A permit applies for those GM plants not meeting the requirements for notification. Examples are if the GM plant species is a noxious weed or if the GM plant species is benign but the genetic alteration results in a PMP. In issuing a permit, BRS is primarily concerned with biosafety, that is, the unintended release and spread of a potential plant pest. The permit procedure[3] is much more elaborate than the notification and requires much more information and data. The application form is available online[4] and can be submitted online via e-permits[5] or manually with hard copy.[6]

In March 2003, in response to concerns surrounding non-food substances in transgenic plants and a series of highly publicized permit violations, APHIS announced that they would strengthen mandatory permit conditions for field-testing transgenic crops, including field trials for PMPs. The number of site inspections would increase to five during the trial and two the following season. The permits for pharmaceutical trials with transgenic maize (a common host plant species) impose several conditions, including that no maize can be grown within one mile of the trial site, that no food or feed crop can be grown on the site the following season and the size of the buffer zone was doubled. For more details on the regulatory aspects governing PMPs, see Stewart and Knight (2005) and Spök et al. (2008).

Deregulation and Commercial Release

In 1992, the USDA proposed regulations to remove regulatory oversight of those GM plants deemed (after appropriate investigation) environmentally benign. In this proposal, GM plant developers could petition the USDA seeking 'non-regulated

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[2] For details on the requirements for the notification procedure, see 7 CFR 340.3.

[3] The regulatory requirements for permits are documented at 3 CFR 340.4, and online information and assistance is available at: http://www.aphis.usda.gov/biotechnology/permits.shtml

[4] Available at: http://www.aphis.usda.gov/brs/pdf/2000.pdf

[5] Available at: http://www.aphis.usda.gov/permits/brs_epermits.shtml

[6] Available at: http://www.aphis.usda.gov/brs/pdf/usersguide.pdf

status', which would then allow commercial release of the deregulated event.[7] The proposal was approved and put into effect in 1993, with the first GM plants achieving non-regulated status within a year. The initial cultivars were a delayed ripening tomato, later known as Flavr Savr, from Calgene, a viral disease resistant squash from Upjohn, a bromoxynil tolerant cotton from Calgene and a glyphosate tolerant soybean from Monsanto. To date, 78 GM plants have achieved non-regulated status via the petition process.[8] The APHIS responses, including the Environmental Assessment (EA), Finding of No Significant Impact (FONSI) and determination of non-regulated status are available on the Internet.[9]

In the process of considering the petition, the USDA prepares at least two documents, an environmental assessment and 'determination of non-regulated status' to satisfy environmental safety issues under PPA and National Environmental Policy Act (NEPA), the latter because, according to the NEPA, the USDA must do an environmental assessment if the GM plant shows potential for 'a significant environmental impact'.

Once an event is deregulated, subsequent events using the same gene construct to transform the same species may be granted a 'fast track' deregulation called an extension. This is somewhat analogous to the Canadian 'Plant with Novel Trait' regulation, in which the agency considers the 'new' event to be sufficiently similar to a prior, approved event that it need not conduct the entire risk assessment. Interestingly, the extension route has rarely been pursued. One prominent exception is for a Bayer Liberty Link rice line (LL601) that was granted deregulation as an extension of a prior deregulation. However, neither rice has been commercialized to date.

National Environmental Policy Act[10]

The National Environmental Policy Act of 1970 requires most federal agencies to investigate environmental impacts prior to making certain decisions or taking certain actions that could pose environmental risks. The relevant agency starts by asking 'Is this decision or action likely to have significant environmental effects?' and then pursues an answer. The simplest finding is a categorical exclusion (CE), which includes items or actions with properties determined by the agency, based on their experience and familiarity, to pose insignificant effect on the environment. After ascertaining that no extraordinary circumstances exist (due to, e.g. possible interactions with unique regional features or endangered species) the agency can approve the application. If the proposal does not warrant a CE or if it may present significant environmental effects, the agency conducts and publishes an EA.

[7] For an example of a petition for non-regulated status for a GM plant under 7 CFR 340, see: http://www.agbios.com/docroot/decdocs/04-225-005.pdf

[8] All of these are documented at: http://www.isb.vt.edu/cfdocs/biopetitions3.cfm

[9] Available at: http://www.aphis.usda.gov/brs/aphisdocs2/98_33501p_com.pdf

[10] The following is a quick review of the NEPA involvement and procedures, but necessarily omits various exceptions, exemptions and appeals procedures. For a comprehensive description, see the NEPA website (www.nepa.gov) or one of the many books on the subject.

The EA is a critical analysis of the environmental consequences of conducting the proposed activity or releasing the item. After reviewing the varied relevant factors, the agency can conclude that either the proposed activity/item demands additional analyses (and issues a Notice of Intent (NOI) to prepare a more elaborate Environmental Impact Statement (EIS)), or that the proposed activity/item poses insignificant risk, and prepares another document, the Finding of No Significant Impact. The FONSI summarizes the EA (or otherwise appended) and justifies and provides rationale, using the data presented in the EA, why the agency came to the conclusion that the activity/item was deemed environmentally benign. Both the EA and the FONSI are public documents and the public has various opportunities to comment and provide input into them.

If the EA suggests the proposed activity or item might present a significant environmental impact, the agency can publish the NOI in the *Federal Register*. The NOI includes information on the proposed activity/item, outlines how the agency plans to proceed with an EIS and how the public can contribute along with contact information at the agency. The plan, also called the 'scoping process', identifies specific relevant issues for in-depth investigation and a time line for completion.

The EIS is a major analysis document, requiring careful deliberation and active wide consultation. When the agency completes a draft EIS, a Notice of Availability (NOA) is published in *Federal Register*, which opens the draft to public comment. For at least 45 days, anyone can read and provide input to the agency, which may additionally provide other fora (such as public meetings) to solicit broad public input. The agency is required to take public comments seriously and respond to all reasonable such input in preparing the final EIS. When the final EIS is completed, the agency publishes another NOA in the *Federal Register*, which signals another 30 day (or more) waiting period before a final decision is made.

Eventually, the agency publishes a Record of Decision (RoD), the final step in the whole process. The RoD summarizes and discusses the issues investigated in the proposed activity/item prior to making the final decision. The RoD is publicly available, but not necessarily published in *Federal Register*.

Current Status

Not everyone agrees the USDA properly follows its own operating procedures. Several recent federal district court suits challenged the USDA for improperly regulating GM plants. Two suits related to field trials (GM herbicide tolerant turfgrass in Oregon and pharmaceutical producing maize and sugar in Hawaii) and one suit related to deregulation of GM alfalfa and one to GM sugarbeet. The USDA lost at trial in each case, with each judge ruling that the USDA was not diligent enough in following the NEPA requirements.

In August 2006, Judge J. Michael Seabright of the Hawaii district ruled that APHIS failed to adequately consider the consequences of allowing field trials of GM maize and sugarcane on the State's many endangered species. On February 5, 2007, Judge Henry Kennedy of the Washington DC district court ruled that

the USDA ignored evidence of potential environmental harm in allowing field trials of GM bentgrass. The following week, US District Judge Charles Breyer in California ruled that the USDA's FONSI decision on GM alfalfa was faulty, because he was not convinced the data in the EA was adequate to reach a FONSI decision. Instead, he ruled, the USDA should have followed the more elaborate and extensive EIS route and ordered an injunction to halt cultivation of the GM alfalfa. Monsanto, developer of the GM alfalfa, appealed through the courts and ultimately, in 2010, the US Supreme Court ruled that the federal court erred in issuing the broad blanket injunction, but did not affirm the APHIS deregulation completely.

More recently, US Federal District Court Judge Jeffery S. White ruled that USDA should have required and conducted an EIS instead of the EA and FONSI when it deregulated Roundup Ready sugarbeets. The ruling effectively canceled the 'deregulated' status and placed the sugarbeet cultivar back into regulated status, meaning it cannot be grown commercially. Although sugarbeet is a smaller crop than alfalfa in the US, the Roundup Ready sugarbeet variety had claimed 95 % market share in its brief commercial release, and this accounts for almost half of the US sugar supply.

The apparent solution to these judgments against USDA is to conduct the full blown EIS on every new regulated article. But this adds considerably to the time and cost of getting an event deregulated, and typically unnecessary, as no GM plants have caused a documented environmental problem since they were first deregulated (under the simpler EA and FONSI) and grown commercially in 1994.

A more expedient solution is for USDA to declare certain categories of GM crops to be exempt from the NEPA requirements. Such categorical exclusions are permissible under NEPA, and justified based on USDA's now longstanding familiarity (since 1988) with GM plants and almost unbroken assignations of FONSIs emanating from EAs dating back almost as many years. While the concept of novelty may have been valid for asserting the regulatory reviews in the 1980s and 1990s, GM plants can no longer be considered categorically 'novel', and USDA is certainly familiar with at least some categories of GM crops. However, USDA has not, to date, taken the categorical exemption approach.

Partly as a result of these various lawsuits against GM crops and the legitimacy of APHIS regulatory procedures, few new GM crop cultivars have been successfully deregulated in recent years. In fact, fewer than 30 GM crop events achieved non-regulated status in the last 10 years, and of those, all but one were bred by a big multinational company (see http://www.isb.vt.edu/cfdocs/biopetitions3.cfm).

Ironically, one of the concerns cited by anti-GM crop activists is their fear that agriculture and plant breeding would become dominated by a handful of large companies. It is largely due to their own litigious and political activities that led to their own nightmare becoming reality. That is, the lawsuits and campaigns against GM crops wrought largely by anti-corporate groups created such high financial and liability cost of regulatory compliance that small companies and public sector breeding institutes could not and still can not afford to participate in the development of improved GM crops, leaving the entire plant breeding effort to the big multinational companies.

3.3.2.2 The Food and Drug Administration (FDA)

The FDA has responsibility for ensuring the safety and security of human food and the supply of animal feed. The Center for Food Safety and Nutrition (CFSAN) and the Center for Veterinary Medicine (CVM) evaluate new GM food and feeds, focusing their attention on food and feed composition, looking for the presence of new or altered allergens and toxicants and changes in levels of ordinarily present nutrients, fiber and other usual constituents.

The FDA likely has the greatest experience dealing with GMOs, starting with the first commercialized GM product, human insulin (FDA approved Genentech's Humulin™ in 1982) and eventually the first food or feed product, Chymosin for cheese making in 1990 (2 years after the same product was approved for commercial release in United Kingdom). The FDA also handled the first approval for a whole GM food product, FlavrSavr tomato, in March, 1994.

In 1992, the FDA issued a policy statement establishing its authority under the Federal Food, Drug and Cosmetic Act (FFDCA, 21 U.S.C. 301) to regulate new food and feeds, irrespective of the method of breeding (FDA 1992). Under this policy, the FDA considers the food or feed composition relative to currently available counterparts, looking especially at the presence of allergens and toxins and any changes in levels of nutritional and anti-nutritional substances. Food containing unexpected or novel substances, or usual substances falling outside normal ranges for that kind of food, are considered 'adulterated' and subject to FDA regulatory action. Food and feeds identical or nearly identical in composition to regular versions are not considered adulterated and do not trigger FDA review, even if they were produced using rDNA technology. The policy states that the FDA is concerned for food and feed safety, and that safety is a function of substances present (or of nutrients absent) from the food in question. If food or feeds produced from or with GMOs are composed of the same substances and in the same amounts and relative proportions, there is no basis for a safety concern (above and beyond whatever safety concerns may ordinarily reside in that food or feed), and no need to invoke the 'adulteration' action trigger. This is why some people consider the FDA review to be 'voluntary'. Because most food and feeds from GM plants are compositionally identical (or nearly so) to regular versions, the FDA does not require mandatory regulatory assessment.

The FDA, in contrast to most other federal regulatory agencies worldwide, which trigger regulatory scrutiny based on the breeding process of GM, regulates food and feeds based on the objective changes in product composition. The FDA agrees with various scientific studies concluding that the process of GM is not inherently hazardous; therefore, the FDA does not compel new food and feeds to undergo regulatory scrutiny due merely to the use of GM breeding methods. The FDA is almost unique in having a scientifically sound basis for its regulatory trigger, recognizing that hazard is due to the presence of tangible substances (or lack thereof), not on breeding method (McHughen 2007).

Although called "voluntary", all GM food and feed currently on the US market has undergone what is called a FDA 'consultation', in which the developer submits a

dossier of compositional data relating to the putative 'identical' food or feed, and FDA scientists evaluate the composition in comparison with the composition of the regular food and feeds. The data submitted include such information as genetic stability of the plant, compositional analyses, nutritional assessment, as well as allergenicity and toxicology of any substances ordinarily present in the food or feed, along with such assessments of the introduced gene products. The FDA published guidelines to assist developers in compiling the dossier in 1997 (FDA 1997). This procedure is beneficial to all parties, as it provides some assurance to consumers that a government agency is evaluating a new food or feed product prior to commercial release. It gives the developer an opportunity to have an independent third party (FDA) cast expert eyes over the data to ensure no potential problems were overlooked, and it keeps the FDA up to speed on new foods and feeds coming through the development pipeline. Even without a compulsion, all developers of GM foods and feeds on the US market have completed the FDA consultation, largely because it is relatively simple, straightforward and prudent to do so. Nevertheless, some people demand that the FDA adjust their policy to make the procedure mandatory. In practice, it already is.

Food and Drug Administration Procedures

Because the FDA consultation is not legally codified, the process is informal relative to the procedures adopted by the other agencies. The FDA's consultation process is concerned with food and feed safety, so the focus is on three initial questions: first, does the new food or feed contain any new allergens?; second, does the new food or feed carry any new toxic substances?; and third, has the new food or feed an altered nutritional composition, such that the usual components are either increased or decreased?

The proponent submits a dossier of data to the FDA consisting of a description of the modified food or feed, and the FDA assigns a caseworker familiar with that kind of food or feed to conduct the consultation. In addition to reviewing the compositional analysis, the caseworker might request information on expected dietary exposure, whether any specific risk groups (i.e. children, elderly, pregnant women, or immunosuppressed patients) might experience increased or decreased dietary exposures, or for a minor food, whether an increased dietary exposure may be experienced by any particular ethnic or religious groups. The FDA will consider both the expected changes in food and feed composition (e.g. the addition of a gene to enhance the levels of a particular nutrient) as well as the possibility that additional levels of this nutrient might result in the decline in levels of other nutrients, especially precursors. Some critics of biotechnology argue that the unexpected changes in foods and feeds are the most worrisome, and such changes may be expected because rDNA is (to them) so 'unnatural' and destructive to the genome.[11]

[11] For examples of such specious arguments, see the website of the Institute of Science in Society (ISIS) at: http://www.i-sis.org.uk/index.php, or that of Jeffrey Smith at: http://www.seedsofdeception.com/Public/Home/index.cfm

It should be noted that the 'unnaturalness' argument has no support from peer reviewed scientific publications, and that these websites and their authors have little or no credibility in the scientific community.

So far, the FDA has not identified any examples of biotech foods with unexpected changes in nutrient composition, or in changes in levels of naturally occurring allergens, toxicants or other anti-nutritional substances ordinarily found in that kind of food (NAS 2004a). Indeed, studies on transgenic wheat show that rDNA transformation causes fewer changes to the plant than are seen in near genetically identical sister lines (i.e. progeny of a cross-pollination with the same parents) that had not undergone rDNA transformation (Baudo et al. 2006; Shewry et al. 2007).

A more legitimate concern – technically – is that the inserted gene produces an allergenic protein. No scientist would consider transferring a known allergenic gene into a food. Fortunately, the chance of unintentionally transferring an allergenic gene is small, as genetic engineers are aware of the issue and seek to avoid using allergenic sources for the genes. In any case, the FDA has allergens at the top of their checklist, so a genetically engineered food carrying a new allergen is unlikely to ever get to market. Indeed, GM breeders developed a soybean carrying an allergenic protein from Brazil nut. The intent was to enhance the nutritional profile of soy using the methionine and cysteine rich storage protein gene from Brazil nut. Researchers did not know at the time that the associated protein was also allergenic. The resulting GM soybean produced the relevant protein and did show the improved nutritional profile, but early testing revealed the allergenic nature of the transferred protein and so the project was terminated well before commercial release (Nordlee et al. 1996).

If such an event were to proceed through the required regulatory reviews and ultimately be commercialized, it would be discovered by the first consumers with the relevant allergy. This would alert the product developer and federal regulators, triggering a product recall, thereby minimizing the potential for adverse health effects. For one thing, the company responsible would face sanctions from the FDA for releasing an adulterated food (according to the definition) but that punishment would likely be insignificant compared to the wrath of litigation from those unsuspecting consumers suffering an allergic reaction from ingesting a previously safe food. With the pragmatic regulatory approach adopted by the FDA, and with the potentially disastrous marketplace consequences of bypassing the 'voluntary' FDA consultation, a GM food developer would be foolish not to seek the FDA's review.

It is worth noting that the FDA does not formally 'approve' an application, or even pass judgment on the safety or efficacy of the new product. Instead the FDA issues a memo summarizing the features and how they may affect safety concerns. The memo indicates that the new food or feed is not materially different in composition or in respect of safety from the unmodified version of the same food or feed. That is, the FDA does not conclude that: 'This new food/feed is safe.' Instead, the FDA concludes, based on evidence reviewed, 'This new food/feed is ***as safe as*** its non-modified counterparts.' To date, the FDA has completed their consultation on almost 100 new GM foods and feeds.

3.3.2.3 The Environmental Protection Agency (EPA)

The EPA enjoys broad regulatory authority over substances with pesticidal characteristics, with particular concern for threats to human health and the environment. In addition to regulating the pesticides themselves, the EPA regulates according to changes in pesticidal properties or pesticide usage. Importantly, the EPA claims not to regulate GM plants *per se*, but rather regulate the pesticidal properties associated with GM plants. This trigger captures plants such as GM virus resistant plants, even though there is no pesticidal substance necessarily sprayed (or synthesized internally), as well as the more obvious herbicide tolerant GM plants, where the crop is designed to be sprayed with a new pesticidal substance, such as the Roundup Ready™ and Liberty Link™ groups of crop cultivars. The EPA also captures GM plants which produce their own substances with pesticidal properties, the plant incorporated protectant (PiP), which means GM plants expressing, for example, *Bacillus thuringiensis* (*Bt*) or other insecticidal substance.

The EPA was given authority to regulate the pesticidal properties in GM plants under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), (7 U.S.C. s/s 135 et seq. 1972) and FFDCA. Under the coordinated framework, the EPA published their proposed regulations in 1994 and began acting on those in 1995. The EPA's working definition of a PiP was '… a pesticidal substance produced in a living plant and the genetic material necessary for the production of that pesticidal substance, where the substance is intended for use in the living plant' (NAS 2000, pg 127).

In 1994, the EPA proposed exempting several low risk categories.[12] One would be those plant pesticides in which the genetic material originates in a sexually compatible species. That is, if the pesticidal trait could be crossed through ordinary breeding, the resulting novel pest protected plant would be exempted under FIFRA. A second exemption category included those using physical barriers (and similar mechanisms such as inactivating toxic substances) to preclude the pest from attaching to or invading the plant. The third category included plants expressing viral coat proteins as means to provide virus resistance. The proposals also included language to circumvent, as required under FFDCA, the establishment of a tolerance limit for such substances (NAS 2000).

By 2001, the EPA issued final rules exempting the previously captured sexually compatible PiPs, as well as exemptions for residues of the pesticidal substances and genetic material (DNA, RNA). The other proposals for exemption remained under review. Recently, the EPA reiterated its desire to exempt virus resistance in plants due to viral coat protein because, with the gain of time and experience lending credibility to the scientific community's prediction that GM plants with these pesticidal properties are unlikely to cause problems, the EPA does not need to routinely capture for full regulatory assessment every similar such plant in future. That is, initially, the EPA invoked the novelty and lack of familiarity of virus resistant viral

[12] Further information is available at: http://www.epa.gov/pesticides/biopesticides/regtools/biotech-reg-prod.htm

coat protein GM plants to capture and assess all such plants prior to commercial release. With the intervening years of experience and familiarity with such products, the exemption proposed in 1994, now has greater credibility. However, EPA has since changed its mind again, requiring for mandatory regulatory review and approval a virus resistant plum developed by USDA- ARS. Such approval, albeit conditional, was finally issued by EPA in 2010, almost 15 years after initial breeding in 1994 (http://www.epa.gov/oppbppd1/biopesticides/ingredients/tech_docs/brad_006354.pdf).

Environmental Protection Agency Procedures

In accordance with the coordinated framework, the EPA evaluates each submission on a case-by-case basis, so the focus of the concerns with novel herbicide uses will differ from those with novel insect protection. To date, all GM PiP plants evaluated by the EPA produce proteins, mainly the *Bt* endotoxin and viral resistance proteins, such as coat proteins. In addition to data requirements related to product characterization, the EPA also requires data on mammalian toxicity, non-target organisms' effects and environmental metabolism. For *Bt* products, the EPA also demands an insect resistance management program. For herbicide resistant GM plants, the EPA coordinates with the USDA and the FDA. The EPA emphasizes that it does not regulate the GM plant *per se*, but the herbicide used on or with the GM plant. For example, with a Roundup Ready™ soybean cultivar, the EPA does not evaluate the soybean plant alone, it evaluates the use of glyphosate (the active ingredient in Roundup™ herbicide) on the new soybean cultivar. Resistance management programs are conducted under a Memorandum of Understanding (MoU) with the other agencies.

The data requirements of the EPA are similar to those of other agencies, notably the USDA and the Canadian Food Inspection Agency (CFIA) as they relate to risks associated with particular substances. The dossier will begin with a description of the plant and its modification. The EPA focuses on the pesticidal properties, so EPA officials need to know the organic source of pesticidal gene construct, along with promoter, enhancer, terminal region, etc. and a description of any marker genes or other segments on the inserted DNA. The biology and any relevant information on the recipient plant species is included, particularly information regarding anti-nutritional substances produced by the plant or its associated pests, pathogens, weeds and relatives. Genetic integrity and stability data on the inserted DNA are required, using molecular techniques, with emphasis on number and location of insertion loci and stability over several seed or vegetative generations.

The pesticidal protein must be fully described (including amino acid sequence) and characterized biochemically, including expression pattern and intensity in various tissues or organs using standardized molecular/biochemical assays. Any modification to the protein, whether intentional (e.g. base changes for codon optimization or amino acid sequence alteration) or unintentional (e.g. glycosylation) need also be reported. Mammalian allergenicity is an issue of concern because most PiPs are proteins and, as most allergens are proteins, gives rise to concerns for allergenicity.

Simple acute digestibility assays and amino acid sequence homology comparisons usually provide sufficient evidence to clear most such proteins from allergenicity concerns, but those failing these tests become subject to more elaborate, longer term immunological or feeding trials. The first step in assessing potential allergenicity is consideration of the species source of the transferred gene. That is, if the source organism is known to produce allergens (e.g. soybean, peanut or fish), that will raise a red flag and justify further investigation. The amino acid sequence of the protein can be searched and compared against known allergens in a database and again, depending on the degree of homology (sequence similarity), the suspect food can trigger greater scrutiny and, ultimately, human trials. Most GM foods do not reach this stage and are either deemed innocuous at an early stage or, if not, dropped from further progression towards commercial release.

Like APHIS at the USDA, the EPA is also concerned with gene flow issues. However, unlike the USDA, where gene flow interest is driven by concern for potential increase in weediness or plant pest characteristics, the EPA's interest in gene flow is due to the possibility of expanding exposure to novel pesticidal substances. The EPA is required by FIFRA to consider adverse environmental impacts attributable to possible gene flow, and by FFDCA to exempt or issue tolerances for the pesticidal substances that might enter the food and feed supply. So far, the EPA has analyzed several plant species with *Bt* constructs and all have received exemptions. The EPA has prohibited the unregulated sale and cultivation of *Bt* cotton, however, in some areas (Hawaii, Florida, Puerto Rico and the U.S. Virgin Islands) due to the local presence of interfertile relatives or feral cotton populations, as they present a recipient sink and opportunity for greater uncontrolled *Bt* exposure.

By the same reasoning, the EPA seeks to preclude gene flow between GM plants and wild or feral relatives as that is a primary means of gene escape, invasion and possible establishment of undesirable plants. To date, this policy has not posed great hardship (except possibly to growers in Hawaii, Florida, Puerto Rico or U.S. Virgin Islands wishing to grow *Bt* cotton) but may take on greater significance with the increasing interest in biofuels made from GM versions of energy crops such as switchgrass. At present, in spite of considerable research and development of technologies to limit gene flow (via, e.g. pollen disabling genes), no such gene flow mitigation technologies is 100 % effective (NAS 2004b).

The EPA is also concerned with effects of PiPs on non-target organisms in the environment. The requirements here involve an initial assessment of potential toxicity and exposure to non-target species, followed, where warranted, by up to four tiers of testing on the relevant species.[13] Finally, the EPA considers the environmental fate of PiP substances, for example of *Bt* endotoxin in the soil, and how soil biota respond to the *Bt* deposited by transgenic plant roots, decaying leaf matter, pollen settling, etc.

The EPA is also concerned about organisms – particularly insects – developing resistance to pesticides, and so the EPA considers management strategies to minimize

[13] Done in accordance to the EPA's Office of Prevention, Pesticides and Toxic Substances (OPPTS) Harmonized Pesticide Test Guidelines.

and delay the onset of resistance in pest populations. Pests are known to develop such resistance to pesticides, antibiotics and other such substances based on exposure and intensity. Because *Bt* is an important insect control chemical to many farmers – even organic farmers – the onset of resistant insect pest populations is a concern for all. The EPA takes the lead in requiring appropriate insect resistance management (IRM) strategies, and farmers are required to follow the IRM practice regulations. For *Bt*, these practices include areas of on farm refugia to allow *Bt* sensitive and resistant insects to mate in the absence of *Bt* selection pressure. The exact size and locations of the refugia will vary depending on the crop, the particular pest and the nature of the pesticide being used. Other factors, such as nearby alternate refugia or PiP crop species, may also influence the optimum presentation of the refugia.

3.4 Summary

The three main agencies identified and given primary responsibility by OSTP for assuring safety of new crops developed by rDNA methods, USDA, FDA and EPA have worked together for over 15 years in conducting scientific risk assessments using their various statutory authorities. Although the US regulatory system is not perfect and can always be improved (see, e.g. McHughen 2007), the US agencies can point to their track record and show that no approved, deregulated GM crop has been recalled or re-regulated due to any documented harm to humans, animals or the environment. This is a remarkable safety record, one enjoyed by no other method of plant breeding.

3.5 Conclusions

The US has an elaborate but coordinated regulatory system to evaluate new rDNA derived crops and foods. The scientific basis for assessing risks combined with the coordinated framework assigning regulatory responsibility gives the US a functional, if imperfect, bureaucracy to allow environmental and market release of agricultural products of biotechnology.

This is not to suggest that the US system is efficient or fair. Indeed, there are substantial inefficiencies and at least one important flaw in the US regulatory system. Most notably, the scientific community both in the US and indeed, around the world, has concluded that using the process of biotechnology as the trigger for regulatory scrutiny is scientifically invalid (McHughen 2007). Instead, regulation should be based on the risks posed by the features of the product, not the process of breeding. The USDA and EPA particularly ignore the findings and recommendations of the scientific community, including many studies and reports from the US National Academy of Sciences, and also ignores its own OSTP by using the process based regulatory trigger, thus unnecessarily imposing significant regulatory requirements

on some non-risky GM plants, and failing to capture occasional risky plants merely because they are not products of biotechnology.

In addition, the current regulatory policies create, perhaps unwittingly, an almost insurmountable barrier to low risk GM plants and foods derived from small market and specialty crops due to the high financial cost of regulatory compliance and the low overall value from the small acreage or small market potential of the special GMOs. That is, the additional market value attributable to the improvements to the GM plant or crop is insufficient to justify the expenditure to meet regulatory demands. This is especially galling for those improvements widely regarded, even in regulatory offices, as being very low risk. Genetically modified plants with considerable health or environmental benefits are denied market access, not because they present undue risk, but because the developer cannot afford to jump unnecessary regulatory hoops that provide little or no confidence in product safety.

Nevertheless, at least some products of biotechnology have passed through the US regulatory bureaucracy since 1994, have been cultivated widely and consumed intensively, and still there are no documented cases of adverse effect on health or the environment from any approved product of biotechnology. Although the rapid adoption of biotech crops by farmers worldwide (NAS 2010; Brookes and Barfoot 2010; James 2009) seems to suggest a potential problem, especially with herbicide resistance and the concomitant inevitability of herbicide resistance, one must place this concern in the context that conventional breeding also generates crops with novel herbicide resistance and, indeed, weeds with resistance to those herbicides. To a large extent, the appearance of weeds acquiring herbicide resistance from GM crops supports and consolidates the early scientific predictions from OECD (1982, 1986), NAS (1983, 1987, 1989) and others that risks associated with GM plants will be the same as those from conventional breeding.

References

Akeley RV, Mills WR, Cunningham CE, Watts J (1968) Lenape: a new potato variety high in solids and chipping quality. Am Potato J 45:142–145

Ames BN, Profet M, Gold LS (1990) Dietary pesticides (99.99 % all natural). Proc Natl Acad Sci 87:7777–7781

Barton KA, Binns AN, Matzke AJM, Chilton MD (1983) Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell 32:1033–1043

Baudo MM, Lyons R, Powers S, Pastori GM, Edwards KJ, Holdsworth MJ, Shewry PR (2006) Transgenesis has less impact on the transcriptome of wheat grain than conventional breeding. Plant Biotechnol J 4:369–380

Berg P, Singer M (1995) The recombinant DNA controversy: twenty years later. Proc Natl Acad Sci 92:9011–9013

Berg P, Baltimore D, Boyer HW, Cohen SN, Davis RW, Hogness DS, Nathans D, Roblin R, Watson JD, Weissman S, Zinder ND (1974) Potential biohazards of recombinant DNA molecules. Proc Natl Acad Sci 71:2593–2594

Berg P, Baltimore D, Brenner S, Roblin RO III, Singer MF (1975) Summary statement of the Asilomar conference on recombinant DNA molecules. Proc Natl Acad Sci 72:1981–1984, also *Science* 188: 991 (1975)

Brookes G (2002) The farm level impact of using Bt maize in Spain. http://www.bioportfolio.com/pgeconomics/spain_maize.htm

Brookes G, Barfoot P (2010) Global impact of biotech crops: environmental impacts, 1996–2008. AgBioForum 13(1):76–94

Cohen SN, Chang AC, Boyer HW, Helling RB (1973) Construction of biologically functional bacterial plasmids in vitro. Proc Natl Acad Sci U S A 70:3240–3244

Food and Drug Administration (1992) Statement of policy: foods derived from new plant varieties. Fed Regist 57:22984–23005

Food and Drug Administration (1997) Guidance on consultation procedures: foods derived from new plant varieties. http://vm.cfsan.fda.gov/~lrd/consulpr.html

Fraley RT, Rogers SB, Horsch RB (1983a) Use of a chimeric gene to confer antibiotic resistance to plant cells. Adv Gene Technol Mol Genet Plants Anim. Miami Winter Symp 20:211–221

Fraley RT, Rogers SB, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffman NL, Woo SC (1983b) Expression of bacterial genes in plant cells. Proc Natl Acad Sci 80:4803–4807

Herrera-Estrella L, Depicker A, van Montagu M, Schell J (1983) Expression of chimeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature 303:209–213

James C (2009) Global status of commercialized biotech/GM crops: 2009 the first fourteen years, 1996 to 2009. *ISAAA Brief* No. 41. ISAAA, Ithaca, NY

James C, Krattiger AF (1996) Global review of the field testing and commercialization of transgenic plants, 1986–1995: the first decade of crop biotechnology. *ISAAA Briefs* 1. ISAAA, Ithaca, NY

Kuiper HA (2003) Biotechnology, the environment, and sustainability. Nutr Rev 61(6: Supplement 1): 105–109

Lindow SE, Panopoulos NJ (1988) Field tests of recombinant Ice-Pseudomonas syringae for biological frost control in potato. In: Sussman M, Collins CH, Skinner FA, Stewart-Tull DE (eds) Release of genetically-engineered micro-organisms. Academic, London

Maluszynski M (1991) Officially released mutant varieties – the FAO/IAEA database. Plant Cell Tiss Org Cult 65:175–177

McHughen A (2000) Pandora's picnic basket: the potential and hazards of genetically modified foods. Oxford University Press, New York

McHughen A (2007) Fatal flaws in agbiotech regulatory policies. Nat Biotechnol 25:725–727

McHughen A, Wager R (2010) Zero sense in European approach to GM. EMBO Rep 11:258–262

National Academy of Sciences (1983) Risk assessment in the federal government: managing the process. National Academies Press, Washington, DC

National Academy of Sciences (1987) Introduction of recombinant DNA-engineered organisms into the environment: key issues. National Academies Press, Washington, DC

National Academy of Sciences (1989) Field testing genetically modified organisms: framework for decisions. National Academies Press, Washington DC. http://books.nap.edu/catalog/1431.html

National Academy of Sciences (2000) Genetically modified pestprotected plants: science and regulation. National Academies Press, Washington, DC

National Academy of Sciences (2002) Environmental effects of transgenic plants. National Academies Press, Washington, DC

National Academy of Sciences (2004a) Safety of genetically engineered foods. National Academies Press, Washington, DC

National Academy of Sciences (2004b) Biological confinement of genetically engineered organisms. National Academies Press, Washington, DC

National Academy of Sciences (2010) Impact of genetically engineered crops of farm sustainability in the United States. National Academies Press, Washington, DC

National Institutes of Health (1976) Recombinant DNA research. Guidelines. Fed Reg 41:131, 27902, 27911–27943

National Institutes of Health (1978) Guidelines for research involving recombinant DNA molecules. Fed Regist 43:60108

Nordlee JA, Taylor SL, Townsend JA, Thomas LA, Bush RK (1996) Identification of a Brazil-nut allergen in transgenic soybeans. N Engl J Med 334(11):688–692

Office of Science and Technology Policy (1986) Coordinated framework for regulation of biotechnology: announcement of policy and notice for public comment. Fed Regist 51:23302–23393

Organisation for Economic Cooperation and Development (1982) Biotechnology, international trends and perspectives. OECD, Paris

Organisation for Economic Cooperation and Development (1986) Recombinant DNA safety considerations: safety considerations for industrial, agricultural and environmental applications of organisms derived by recombinant DNA techniques (*aka "Blue Book")*. OECD, Paris

Pew Initiative on Food and Biotechnology (2004) Genetically modified crops in the United States. www.pewagbiotech.org

Schell J, van Montagu M, Holsters M, Zambryski P, Joos H, Inze D, Herrera-Estrella L, Depicker A, de Block M, Caplan A, Dhaese P, Van Haute E, Hernalsteens JP, de Greve H, Leemans J, Deblaere R, Willmitzer L, Schroder J, Otten L (1983) Ti plasmids as experimental gene vectors for plants. Adv Gene Technol Mol Genet Plants Anim. Miami Winter Symp 20:191–209

Shewry PR, Baudo M, Lovegrove A, Powers S, Napier JA, Ward JL, Baker JM, Beale MH (2007) Are GM and conventionally bred cereals really different? Trends Food Sci Technol 18(4):201–209

Smyth S, Phillips PWB, Kerr WA, Khachatourians GG (2004) Regulating the liabilities of agricultural biotechnology. CABI Publishing, Wallingford

Smyth SJ, Endres AB, Redick TP, Kershen DL (2010) Innovation and liability in biotechnology: transnational and comparative perspectives. Edward Elgar Publishing, Cheltenham

Spök A, Twyman RM, Fischer R, Ma JKC, Sparrow PAC (2008) Evolution of a framework for plant-made pharmaceuticals. Trends Biotechnol 26(9):506–517

Stewart PA, Knight AJ (2005) Trends affecting the next generation of U.S. agricultural biotechnology: politics, policy, and plant-made pharmaceuticals. Technol Forecast Soc Change 72:521–534

Zitnak A, Johnston GR (1970) Glycoalkaloid content of B5141-6 potatoes. Am Potato J 47:256–260

Chapter 4
Regulation of Genetically Engineered Microorganisms Under FIFRA, FFDCA and TSCA

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Abstract Since the dawn of civilization, humans have utilized microbial organisms of various sorts for food and agricultural production. More recently, microbes have been used for pesticidal, and environmental management purposes. With the advent of the development of recombinant DNA technology to genetically alter microbes, it became necessary for Federal regulators to assess the appropriate level, format, and application of their regulatory authorities. In 1986, the Office of Science and Technology Policy issued the Coordinated Framework for Regulation of Biotechnology. The Coordinated Framework constituted a comprehensive regulatory policy for biotechnology that, in essence, concluded that no new statutory authorities were necessary to effectuate a robust and efficient regulatory program for the products of biotechnology. The Framework articulated a division of regulatory responsibilities for the various agencies then involved with agricultural, food, and pesticidal products. Thus, in accordance with the Framework, USDA APHIS regulates microbes that are plant pests under the Plant Protection Act (PPA) and the National Environmental Policy Act (NEPA); the U.S. Environmental Protection Agency (U.S. EPA) regulates microorganisms and other genetically engineered constructs intended for pesticidal purposes and subject to the Federal Insecticide Fungicide and Rodenticide Act (FIFRA) and the Federal Food Drug and Cosmetic Act (FFDCA). The U.S. EPA also regulates certain genetically engineered microorganisms used as biofertilizers,

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bioremediation agents, and for the production of various industrial compounds including biofuels under the Toxic Substances Control Act (TSCA). The focus of this chapter is the regulatory process for approval of the use of genetically engineered microbes under the oversight of the U.S. EPA. We will also consider instances where organisms may be exempted from oversight and the outlook for the application of GE microbes in the future. This chapter does not seek to serve as a guidebook for navigating the details of the regulatory process, but rather as an overview of key considerations in risk assessment and risk management.

Keywords Algae • Bacteria • Baculovirus • Biofertilizer • Biofuel • Biopesticide • FFDCA • FIFRA • Fungi • Genetically engineered • Microorganism • MPCA • Plant pest • Plant protection act • Regulation • TSCA

Disclaimer

The content of this chapter reflects the opinions of the authors and this chapter is not intended to constitute a statement of the official policy or actions of the U.S. Environmental Protection Agency.

4.1 Introduction

4.1.1 Historical Regulatory Perspective

The regulation of products of biotechnology has a lengthy history in the United States. Prior to the development of a formal regulatory structure, many protracted discussions took place for well over a decade among scientists, government regulators, environmental activists, and representatives of industry (Berg and Singer 1995; Barinaga 2000). These discussions eventually resulted in the announcement by the Office of Science and Technology Policy (OSTP) of the Coordinated Framework for Regulation of Biotechnology (OSTP 1986).

In February 1975, the Asilomar Conference was convened with 140 scientists, lawyers, physicians, ethicists and other interested parties in Monterey, CA for a comprehensive discussion of the issues surrounding the release of genetically engineered organisms into the environment. A growing sense of concern was mounting among scientists regarding this new ability to reshuffle DNA between microbial agents and this was a major impetus for the conference. While a formal regulatory system would have to wait for further executive and legislative decisions, the Congress at Asilomar helped focus the publication of the National Institutes of Health (NIH) Recombinant DNA Guidelines in 1976 (NIH 1976), even though this project was already underway (Marchant 1988). The primary utility of the 'NIH Guidelines', as they came to be known, related to confined applications, e.g., laboratory

work for research purposes. Recognizing, however, that the NIH Guidelines did not provide genuine oversight for actual environmental releases of GE microbes, Federal regulatory agencies were considering appropriate means of adequately regulating such releases. (It should be noted that the NIH Guidelines are still in effect, with some modifications over the years, for their original intended purpose; NIH 2011).

The principal tenet of the Coordinated Framework was that existing statutes were sufficient to effectuate proper regulation of the products of agricultural biotechnology, i.e., that it was not necessary to legislatively create new statutory authorities specifically for the governance of products in the research pipeline and those that where then envisioned. Existing statutes were considered as a sound basis for oversight of biotechnology with modifications offered through promulgation of regulations via rulemaking.

Given the plethora of potential products to be derived from rDNA technology, the U.S. government was faced with the application of statutes already in use for regulation of pesticides (i.e., FIFRA), plant pests (i.e., Plant Pest Act) and pesticide residues on food and feed commodities (i.e., Federal Food Drug and Cosmetic Act) with implications for the associated agencies, EPA, USDA-APHIS, HHS-FDA, respectively. There were, of course, dissenting views as to whether relying on existing statutes was either sufficient or preferable with regard to necessary regulatory authorities applicable to these technologies and resulting products (Jones 1999).

Environmental releases of genetically modified organisms were proposed for the first time nearly simultaneously by Monsanto and Advanced Genetic Sciences, Inc. (AGS) (Watrud et al. 1985; Lindow 1985). AGS developed a product named FrostBan®, a *Pseudomonas syringae* engineered such that a gene coding for a protein necessary for ice-nucleation had been deleted, and conducted a field release on strawberry fields in University of California experimental plots under EPA and California Department of Food and Agriculture authority on April 24, 1987 (Smith 1997).

Initial approval granted by the NIH administrator (48FR9436; 48FR:24548) for this field test was overturned due to a May 16, 1984 decision (OTA 1988) that the environmental impacts under NEPA were not assessed, though the decision also affirmed that field testing could take place once an environmental effects assessment was performed (Pizzuli 1984). Through a series of events EPA was assigned the task of assessing environmental impacts, though the permit was withdrawn just prior to the 1987 field test when another test, this one an experimental rooftop injection of Frostban® into trees, was declared in violation of the issued permit resulting in a $20,000 fine – though AGS claimed the bacterium injected into trees was a contained use (New Scientist 1986). A field test of Frostban® on strawberry plants did occur at Conta Costa, CA following Federal and State approvals (Supkoff et al. 1988). Steve Lindow of the University of California at Berkeley also conducted frost prevention tests with his deletion mutant IceMinus *Ps. syringae* on potato plants at Tulelake, CA despite some vandalism by opponents of GE technology and a lengthy permitting process (Maugh 1987).

A subsequent genetically engineered construct involved transformation systems directing placement of *Bacillus thuringiensis* (B.t.) transgenic sequences into the bacterial chromosomes of *Clavibacter xyli* ssp. *cynodontis* and *Pseudomonas*

fluorescens, respectively. A system devised by Crop Genetics International (CGI) focused on delivery of the B.t. Cry1Ac δ-endotoxin in tissues of maize by introducing genetically modified *C. xyli* into maize xylem vessels (Turner et al. 1991). *C. xyli* is a natural endophyte of Bermuda grass, maize and several other plants, hence, its potential as a delivery agent of a biopesticidal protein was sought as a means of reducing feeding damage to corn earworm and European corn borers and as a way to reduce environmental exposure to non-target organisms (Lampel et al. 1994). Due in part to the overall concentration of the B.t. δ-endotoxin contained in the endophytic populations of *C. xyli* in maize, this construct ultimately failed to consistently deliver sufficient control during field trials. In addition, there were serious concerns about the possible uncontrolled spread of the genetically engineered microorganism to other plants. Yield was also affected in some maize varieties because of occlusion of xylem vessels with bacteria, particularly when drought stress was an issue (John Turner, personal communication 2011). Regulatory costs associated with field release permits (USDA-APHIS) and experimental use permits (US EPA) were a factor for CGI in that they were a relatively small company without a broad portfolio of products. In 1994, further research into this mechanism of delivery into maize was halted by CGI (Wrubel et al. 1997).

Monsanto's approach was to create an insecticidal, plant rhizosphere dwelling microbe by cloning the *Bacillus thuringiensis* subsp. *kurstaki* HD-1 crystalline protein gene into strains of *Pseudomonas fluorescens* (Obukowicz et al. 1986). Limited field releases of these live microorganisms occurred, though only with strain variants engineered with reporter genes (Kleupfel et al. 1991; Angle et al. 1995; Gagliardi et al. 2001). EPA questioned the safety of pseudomonads expressing B.t. endotoxins in aquatic environments, and this led to Monsanto's decision to cease work on use of engineered microbes as pesticides. Subsequent work in contained settings has shown that runoff from simulated agricultural plots containing *Pseudomonas chlororaphis* (*aureofaciens*) 3732 can be significant (Gillespie et al. 1995), and the general lack of available non-target aquatic invertebrate tests to evaluate such effects leaves regulatory certainty for this use in limbo.

Subsequently, between 1991 and 1996, four genetically engineered microbial preparations were registered under FIFRA as encapsulated *B. thuringiensis* δ-endotoxins in killed *Pseudomonas fluorescens*. Delivery of the B.t. δ-endotoxin in killed *Pseudomonas* had a distinct advantage over using live *B. thuringiensis* in that higher levels of toxins are produced by the pseudomonads during fermentations and some protection against UV light inactivation of the toxin was gained via encapsulation within the killed pseudomonad cell wall (OTA 1995; Mycogen 1998; Shand 1989). Additionally, the use of killed bacteria as the end product alleviates any concerns over spread and reproduction of the engineered pseudomonad; this was a consideration by both the company and EPA risk assessors (BLR 1988).

In addition to the transgeneric expression of B.t. δ-endotoxin genes in the heat-killed pseudomonads, creating a so called 'killed microbial' pesticide, several companies moved forward with engineering of *B. thuringiensis* strains directly, either modifying native *cry* gene sequences or adding to the resident *cry* genes with additional

cry genes in order to broaden the range of susceptible insect species (Baum et al. 1996; Sanahuja et al. 2011).

In addition to these pseudomonad constructs, six submissions were received by EPA for field testing of genetically modified baculoviruses from May 1995 through August of 1998. Four of these utilized the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) with additions of insect-specific toxin genes: three from two different scorpions (Summers 2006) and one from a mite (Tomalski et al. 1989). Two others are based upon modified *Helicoverpa zea* single-embedded nuclear polyhedrosis virus (HzSNPV) each using an insect-specific scorpion toxin from one of two scorpion species. Since the main issues are very similar between the various baculovirus constructs, only a few examples will be discussed in detail herein.

Work with engineered baculoviruses was quite active in the 1990s (Hughes et al. 1997), and even earlier in the UK (Bishop 1988) for control of insect pests on vegetables, ornamentals, and in forestry situations, and some of this work continues today (Tang et al. 2011). Much of the effort centered on addition of scorpion toxin genes to enhance the kill rate of AcMNPV and HzNPV without a consequent change in host range. Toxins from both *Leiurus quinquestriatus hebraeus* (Israeili yellow scorpion; LqhIT2) and *Androctonus australis hector* (Algerian scorpion; AaIT) were used by American Cyanamid and DuPont in an attempt to increase mortality in the target pest without altering the risk profile for non-target species that may feed on the infected insect pests (Bill Schneider, Personal Communication 2010; Gard et al. 2002; Heinz et al. 1995; American Cyanamid 1994, 1996; DuPont 1996; Kunimi et al. 1996).

These scorpion toxins act through either a depressant (LqhIT2) or stimulant (AaIT) capacity on neurons through sodium channel modulation, however, they do not have demonstrable vertebrate activity nor do they affect Crustacea (Hoover et al. 1996; Gard et al. 2002). EPA required testing of a range of surrogate species, including rats, Bobwhite quail, Mallard ducks, rainbow trout, and grass shrimp, which were fed infected *H. zea* larvae. Additional tests with NPV occlusion bodies (OBs) suspended in aqueous media indicated a lack of pathogenic or toxic effect on *Daphnia magna*, the water flea. Testing of human cell lines (liver, lung, intestine) was also performed with budding virus particles with no indication of alterations to cell morphology or timing of division. It is noteworthy that although guidance on assessing human health and environmental risks has adapted to newer technologies as they arose, many of the principals have been in place prior to the advent of biotechnology and rDNA methods (Engler 1974).

Additionally, the ecdysteroid UDP-glucosyl transferase gene (*egt*) had been found to alter ecdysoid hormone levels and influence killing rate, feeding period and molting of several insect species (O'Reilly and Miller 1989, 1991; Slavicek et al. 1999). Removal of the *egt* gene from the AcMNPV genome resulted in feeding cessation and wandering behavior of infected larvae, which succumbed to the viral infection prior to pupation. The combination of the AcMNPV/LqhIT2 toxin and deletion of *egt* resulted in a higher mortality rate during initial measurements soon after infection experiments comparing recombinant strains to wild type AcMNPV,

however, following extended incubation (e.g. a few days post infection to as many as 21 days depending on the virus:insect combination), mortality was equal between the two groups. The titer of occlusion bodies present in the AcMNPV/LqhIT2 strain was, however, significantly less than wild type infections (Tomalski and Miller 1991; Cory et al. 1994). Depending on the strain of virus and the intended host, reductions in yield of virus have varied from 30 to 50% and the rate of kill increased by as much as 95% (Cory 2000). The decreased viral load following infection and the limited host range of most baculoviruses fit prominently into the EPA's risk assessment for these modified biopesticides. The inability of these genetically engineered baculoviruses to persist in the environment and potentially exchange genes with wild type strains or related viruses reduced the uncertainty associated with field release of constructs previously evaluated in laboratory settings (OSTP 2001).

Another consideration of the risk assessment for AcMNPV/LqhIT2 and other recombinant baculoviruses was whether these novel strains could outcompete and eliminate wild type viruses over time. In addition to the noted decrease in viral load following host mortality, experiments and observations demonstrated that larvae infected with AcMNPV expressing insect-specific toxins were susceptible to 'knockoff' wherein they would drop from plant surfaces hours earlier than wild type infected larvae, thereby limiting spread of the OBs onto leaf surfaces where they may contact other larvae (Inceoglu et al. 2006). Further experiments with combinations of GE and wild-type NPVs also indicated that sequential passage to larval hosts resulted in the eventual elimination of the toxin expressing virus strains. In some instances, the GE baculoviruses were comparable in efficacy to conventional insecticides with a 30–40% increase in the speed of killing larvae as compared to non-GE baculoviruses (Hoover et al. 1996).

Shortly after the initial proposed field releases, non-pesticidal uses of genetically modified microorganisms began to be developed. By 1987 initial releases of *Ensifer* (*Rhizobium*) *meliloti* were under TSCA review and initial experimental releases took place by 1988 (EPA 1999). The Monsanto *Pseudomonas chlororaphis* (*aureofaciens*) strain containing reporter genes was also submitted for TSCA review in 1987 and went to the field that same year.

While this chapter considers the oversight of GE microorganisms by the US EPA, it should be noted that some of these organisms may also be regulated by the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS). Both the Plant Protection and Quarantine (PPQ) and the Biotechnology Regulatory Services (BRS) divisions within USDA-APHIS may be involved in the importation, movement and field release of non-GE and GE microorganisms under the Plant Protection Act and National Environmental Policy Act (OSTP 2001). The Food and Drug Administration reviews all genetically engineered microorganisms that may cause an alteration in the nutritional state of a food, or otherwise contribute to a food safety issue. When in doubt as to which agencies may exercise regulatory authority over a particular microbe and its intended use, it is best to contact the agency directly for clarification.

4.2 FIFRA Risk Assessment of Genetically Engineered Microbial Pest Control Agents

Under FIFRA, microbial biopesticide products, as with all other pesticides, must be evaluated for their risks and benefits. Bacteria, viruses, protozoa, algae, and fungi intended for use as pesticides are regulated under FIFRA by the US EPA (40 CFR Part 158.2100). Additionally, the Agency evaluates the potential for effects upon threatened and endangered species under the Endangered Species Act, but this will not be discussed further in this section. There are three principal sections to the FIFRA risk assessment for genetically engineered microbial pest control agents (GE-MPCA): product analysis, human health, and environmental considerations (McLintock et al. 2000). The aim of this chapter is not to consider the data requirements associated with these sections in great detail, but, rather, to present an overview of key considerations. One important note: EPA evaluates an MPCA using the same data requirements, regardless of whether it is genetically engineered or naturally occurring (Baum 1998).

Under the product characterization section (40 CFR Part 158.2120) of the data requirements a summary of the taxonomy, natural history, target, and non-target host range is required. For any genetically modified MPCA, the product analysis portion of the data requirements seeks to provide the risk assessor with necessary information regarding the nature of the transformation event and includes DNA sequences of transgenes, associated vector sequences with restriction map, DNA source information and an indication of transgene stability over multiple generations or growth cycles (e.g., 5 batch analysis). Also critical to this section is the Confidential Statement of Formula, which details the active ingredient(s), inert ingredients, and concentration of the MPCA in its final product formulation. Any pesticide in use under a FIFRA Section 5 Experimental Use Permit, or Section 3 Registration, which is not in accord with the information present on the CSF is considered as ‘Misbranded’ and therefore illegal (FIFRA 2(q)).

Toxicology data requirements (40 CFR, Part 158.2140) explore the potential impact of the MPCA on humans in terms of toxicity, infectivity and pathogenicity. The MPCA is introduced via oral, pulmonary, and injection (intravenous or intraperitoneal) routes into rodent test animals functioning as surrogates. Animal body and organ weights, behavior, and mortality are all assessed as part of these studies, but most important is establishing clearance of the MPCA from the body over time. These high dose tests (at least 10^8 units of the MPCA per test animal) are intended to examine the outcomes following a single, significant contact with an MPCA by various exposure routes (mouth, nose, lungs, and dermal).

Non-target organism and environmental fate data requirements (40 CFR, Part 158.2150) evaluate the potential for the MPCA to impact organisms beyond the intended target pest(s). These studies require examination of pathogenicity on related (e.g., other insects) and unrelated (e.g., plants, birds, and mammals) organisms. The organisms chosen for study are functioning as surrogates, representative of broader groupings (e.g., Mallard duck for birds in general), and include wild mammals, birds, fish, beneficial insects, aquatic invertebrates, estuarine and marine organisms

(fish and invertebrates), plants, and honeybee testing. In accord with 40 CFR, Part 158.30, the Agency has flexibility in determining which of these data requirements must be in the form of generated data or related information, and which can be satisfied by waiver rationale.

The Environmental Fate data requirements focus on the fate of the organism in the area of application to determine the ability to persist and where the organism exists (e.g., in soil, associated with insects, etc.). The survivability and host range of an organism are key to understanding the ability of an MPCA to persist in the environment and potentially result in adverse effects (Hu and St. Leger 2002; 40 CFR 172.45(e)). For example, release of entomopathogens may require monitoring of resident arthropods to determine the ability to colonize and infect as a means of assessing persistence (St. Leger et al. 1996). Reproduction (e.g., sporulation) on cadavers of target hosts or lack thereof can be helpful in ascertaining the ability of the MPCA to persist following small scale release. Rhizospheric competence was also assessed with another set of constructs in *M. anisopliae* (now *M. robertsii,* J.F. Bisch., Rehner & Humber) as part of an investigation into survivorship in the environment (Hu and St. Leger 2002).

As with all pesticides applied to food or feed crops, a food tolerance or the exemption from the requirement of a food tolerance must be in place if any residues of the pesticide may be present on any food derived from the crop. In all cases to date, the MPCAs registered by the Agency have been granted an exemption from the requirement for a tolerance based upon a determination that there is a reasonable certainty that no harm will result from dietary exposure to the MPCA. In general, pesticides containing elements of any of the eight major allergens are not approved for use on most food or feed crops, which could also extend to any expressed proteins originating from peanuts, tree nuts, milk, soybeans, eggs, fish, Crustacea, and wheat (40CFR 180.950).

While the same set of data requirements are imposed upon naturally occurring and GE microbial agents, genetically modified MPCA and non-indigenous microbial species may be subject to additional data or information requirements on a case-by-case basis depending on the particular microbial agent and/or its parental strains, the proposed pesticide use pattern, and the manner and extent to which the organism has been genetically modified (FR 2007).

4.2.1 Biotechnology Notification Process for Microbial Pest Control Agents

At least 90 days prior to conducting any small scale test of a genetically modified microbial pesticide, other than those described at 40 CFR 172.45(d), a Notification must be submitted to the EPA in which the details of the genetic modification, proposed application methods and sites, and any potential toxicity or non-target organism effects are delineated. 40 CFR 172, subpart C. Measures must also be outlined in the Notification submission which indicate the methods of containment and monitoring used to ensure the GEO does not become established in the ecosystem. 40 CFR 172.48. The data required to support a request for a Notification are detailed in 40 CFR Part

172.48 (FR 59, 169, Sept. 1, 1994). If the proposed field test is to be greater than10 acres of treated land per pest evaluated or greater than1 acre, for aquatic uses, then an experimental use permit is necessary. 40 CFR 172.3.

Under FIFRA, a Biotechnology Notification Process (40 CFR, Part 172.43; BNP) for release of a GE-MPCA at any size test plot requires review and approval by the EPA prior to commencing experimentation. EPA requires notification prior to small scale field testing of genetically engineered and non-indigenous microorganisms not subject to USDA oversight to allow EPA to determine if an Experimental Use Permit is needed and to allow the applicant to gather data critical to the risk assessment process. Processing times for review and approval of BNP applications are considerably shorter than those encountered with Experimental Use Permits (EUPs) and Section 3 registrations, and they are intended for smaller (e.g., ≤1 A) field test plots than EUPs. It must be emphasized that with BNP approvals, any treated plants or materials are prohibited from entry into the food and feed supply unless a food tolerance or exemption from the requirement of a food tolerance under Section 408 of FFDCA is in place; these environmental releases are strictly for research purposes only. The treated produce of a BNP or EUP may be allowed for consumption by experimental animals, however, the products of those animals are not allowed for entry into the food or feed supply unless an appropriate food tolerance action is in place.

Several GE MPCA have been through the BNP successfully and field tested on a small scale (See Table 4.1). This includes the first approved field test of a GE microbe, strains of *Pseudomonas syringae* and *Erwinia herbicola* with an ice-minus phenotype applied to potatoes as a means of preventing frost and its associated plant damage (Lindow and Panopoulos 1988; Milewski 1987). Advanced Genetic Sciences (AGS) had engineered a *Ps. syringae* resulting in the absence of expression of a membrane protein responsible for ice nucleation, though the product currently marketed as 'Frostban®' is not genetically engineered and is a naturally occurring ice-minus strain. Another wildtype ice+strain of *Ps. syringae* is also marketed, as 'Snowmax' and is utilized in artificial snow-making operations, however, it is not regulated as an MPCA.

Other successful BNP environmental releases include two *Metarhizium anisopliae* strains modified to enhance virulence through addition of native protease genes (St. Leger et al. 1996) and, in a separate BNP, a gene derived from the scorpion *Androctonus australis* encoding a known neurotoxin active against tobacco hornworm (Wang and St. Leger 2007).

4.2.2 Experimental Use Permits for Microbial Pest Control Agents

When testing a MPCA at 10 acres or more (1 A or more for aquatic use), EPA requires an Experimental Use Permit before field testing naturally occurring or genetically engineered MPCA (40 CFR Part 158.2170; 40 CFR Part 172.3). EUPs for GE-MPCA typically involve larger acreages than those approved under a BNP; however, pesticide

Table 4.1 EPA/FIFRA regulation of genetically engineered organisms for environmental release

Year	Organism	Trait	Intended use/ref	Regulatory action	Registrant
1985, 1987	*Pseudomonas syringae* RGP36R2 and *Ps. fluorescens* GJP17BR2	Ice minus (absence of membrane protein inducing crystallization of water)	Frost prevention Smith (1997)	Biotech notification	Advanced Genetic Sciences
1987	*Pseudomonas syringae*	Ice minus (absence of membrane protein inducing crystallization of water)	Frost prevention Lindow and Panopoulos (1988)	Biotech notification	University of California – Berkeley
1988	*Clavibacter xyli* subsp. *cynodontis*	Insecticidal Cry toxin from *B. thuringiensis var. kurstaki*	Insecticide Turner et al. (1991)	Experimental use permit	Crop Genetics International
1987 1990	*Pseudomonas fluorescens*, M-Cap™ [b]	CryI proteins from *B. thuringiensis* var. *san diego*	Insecticide	Experimental use permit Registration	Mycogen
1990, 1991, 1994	*Pseudomonas fluorescens*, MVP®[b]	Chimeric Cry1Ac/Cry1Cproteins from *B. thuringiensis* var. *kurstaki*	Insecticide Navon (2000)	Experimental use permit Registration	Mycogen
1991	*Pseudomonas fluorescens*, M-One Plus[b]	CryI proteins from *B. thuringiensis* var. *san diego*	Insecticide	Registration	Mycogen
1992	*Pseudomonas syringae* 742RS/*Ps. fluorescens* A506 and 1629RS	Ice minus (absence of membrane protein inducing crystallization of water)	Frost prevention	Registration	Frost Technology Corporation Plant Health Technologies
1993 1995	*Pseudomonas*[b] *fluorescens*, Mattch™	Cry IA(c)/CryIA(b) and CryIC/CryIA(b) chimeric proteins from *B. thuringiensis* var. *kurstaki*	Insecticide Federal Register (1995)	Experimental use permit Registration	Mycogen
1993	*Bacillus thuringiensis*, ECX9399	Cry proteins from *B. thuringiensis* var. *kurstaki*	Insecticide All et al. (1994)	Experimental use permit	Ecogen
1994, 1996	Nuclear polyhedrosis virus of *Autographa californica* AcMNPV	Insecticidal toxin AaHTI from *Androctonus australis hector*	Insecticide	Biotechnology notification	American Cyanamid

1995, 1996, 1997, 1998	Nuclear[a] polyhedrosis virus of *Autographa californica* AcMNPV	Insecticidal toxin LqhIT2 from scorpion *Leiurus quinquestriatus hebraeus*	Insecticide Federal Register (1997)	Biotechnology notification	American Cyanamid
1995	*Bacillus thuringiensis* EG7673 Raven™ OF	Cry 3Bb and 3Aa proteins from *B. thuringiensis* var. *tenebrionis*	Insecticide Baum (1998)	Registration	Ecogen/Certis
1996	*Bacillus thuringiensis*, EG7841 Cry Max® WDG/WP	Cry 1C proteins from *B. thuringiensis* var. *aizawai*	Insecticide Baum (1998)	Registration	Ecogen/Certis
1996, 1997	*Helicoverpa zea* single-embedded nuclear polyhedrosis virus HzSNPV	Insect-specific toxin from the venom of the scorpion *Leiurus quinquestriatus hebraeus*	Insecticide Federal Register (1997)	Biotechnology notification	DuPont
1996	Nuclear polyhedrosis virus of *Autographa californica* AcMNPV	Insecticidal toxin (TxP-I) from *Pyemotes tritici*, straw itch mite	Insecticide Tomalski and Miller (1991)	Experimental use permit	American Cyanamid
1996	Nuclear polyhedrosis virus of *Autographa californica* AcMNPV	Insecticidal toxin (TxP-I) from *Pyemotes tritici*, straw itch mite	Insecticide Tomalski and Miller (1991)	Experimental use permit	DuPont
1996, 1997	Nuclear[a] polyhedrosis virus of *Autographa californica* AcMNPV	Insecticidal toxin from *Leiurus quinquestriatus hebraeus*	Insecticide Federal Register (1997)	Biotechnology notification	DuPont
1997	*Bacillus thuringiensis* EG7826 Lepinox™ WDG/G	Cry 1Ac/1 F[c] protein from *B. thuringiensis* var. *kurstaki*/*aizawai*	Insecticide Baum (1998)	Registration	Ecogen/Certis

(continued)

Table 4.1 (continued)

Year	Organism	Trait	Intended use/ref	Regulatory action	Registrant
1997	*Helicoverpa zea* single-embedded nuclear polyhedrosis virus HzSNPV	Insecticidal toxin from *Androctonus australis* AaH IT1 and prevent expression of the ecdysteroid UDP-glucosyltransferase gene (*egt*)	Insecticide	Biotechnology notification	American Cyanamid
1997	*Pseudomonas fluorescens*, M-Press®	Chimeric Cry1F/Cry1A(b) toxin from *B. thuringiensis* var. *aizawai*	Insecticide	Experimental use permit	Mycogen
1997	*Rhizobium* and *Sinorhizobium*	Expressing genes for trifolitoxin to outcompete soil bacteria and a hydrogenase for enhanced nitrogen fixation	Bactericide Federal Register (1997)	Biotech notification	Eric Triplett, University of Wisconsin-Madison
1998 2002	*Escherichia coli* K-12 derivative; heat-killed	Harpin protein from *Erwinia amylovora*	Plant disease prevention	Experimental use permit Registration	EDEN Biosciences Corp.
1999	*Agrobacterium radiobacter* K1026	Removal of DNA transfer function by deletion	Bactericide, Plant disease prevention	Registration	Bio-Care Technology Pty Limited
1999	*Metarhizium anisopliae*, ARSEF 1080	Enhanced protease production, Pr1 gene from *M. anisopliae*	Insecticide St. Leger et al. (1996)	Biotechnology notification	Ray St. Leger, Univ. of Maryland
2001, 2004	*Pichia pastoris*; heat killed	Insecticidal Trypsin Modulating Oostatic Factor	Larvacide for mosquitoes	Biotechnology notification, Registration (Technical Product only)	Insect Biotechnology, Inc.
2004	*Cryphonectria parasitica* ATCC 38755 ATCC 64671	Reduced virulence gene from mycovirus CHV1-Euro7	Fungicide/Protectant	Biotechnology notification	West Virginia University

2005	*Escherichia coli* K-12 derivative; heat-killed	Harpin αβ protein with components of *hrpN* and *hrpW* (*Erwinia amylovora), popA (Ralstonia solanacearum),hrpZ (Ps. syningae)*	Plant disease prevention, growth enhancement	Registration	EDEN Biosciences Corp.
2007	*Metarhizium anisopliae*, ARSEF 549	Scorpion toxin from *Androctonus australis* AaIT	Insecticide	Biotechnology notification	Ray St. Leger, Univ. of Maryland
2011	Citrus tristeza closterovirus	Anti-microbial peptides	Bactericide, Plant disease prevention	Biotechnology notification	Southern Gardens Citrus, University of Florida
			Folimonov et al. (2007)		

[a]See SIDEBAR No. II.A, BIOCONTROL USING A VIRUS (AcMNPV) following the Bt-maize case study. http://www.whitehouse.gov/files/documents/ostp/Issues/ceq_ostp_study3.pdf. Accessed 22 Nov 2010

[b]Cry proteins expressed in *Ps. fluorescens* cells, cells then killed prior to application

[c]Cry fusion protein

products used under an EUP also require an approved label for experimental use and interstate shipment (40 CFR Part 172.6); this is not the case for BNP testing. Several genetically modified biopesticides have been approved for use under EUPs; but, a number of these were never actually applied or in some cases only sparingly applied (Table 4.1). The reasons for this relate to issues of public perception (i.e., is the company or researcher willing to deal with public meetings and scrutiny?) and business decisions (e.g., is there sufficient market potential to warrant the development and regulatory costs?).

The data requirements for an EUP involving GE-MPCA are discussed at 40 CFR Part 174.3 and the specific tests, also germane to non-GE MPCA, are described in 158.2171–158.2174. In general, the data requirements for an EUP or Section 3 registration are similar, however, the limited exposure to the environment from the small scale field testing of an MPCA under an EUP does not require the same level of non-target organism testing as when full commercial registration is approved through registration procedures. This is due in large part to the limited scope of the environmental release at the EUP stage and the fact that much of the non-target effects information may be collected as part of the EUP overall plan.

4.2.3 Section 3 Registration of Microbial Pest Control Agents

Before any microbial pesticide registration is granted under FIFRA, EPA considers such issues as potential adverse effects to non-target organisms, environmental fate of the microorganism, and the potential toxicity, pathogenicity and infectivity of the microorganism to humans and other animals. These issues are the same as those considered for non-engineered microbial agents approved for pest management, and reflect the inherent similarities of the functional properties of the organism regardless of whether the traits of primary interest are derived from rDNA or not.

The data requirements for registration of a microbial biopesticide are delimited in 40 CFR 158.2120–158.2150. The data and information garnered from the fulfillment of these data requirements are used to inform the risk assessment process, just as with the EUP and BNP applications. All of the data requirements must be satisfied for a FIFRA Section 3 registration, however, in some instances rationale can be provided by the registrant to explain why the requirement is not applicable to the MPCA in question. For example, a psychrotropic bacterium which does not grow at temperatures greater than 20 °C is unlikely to result in mammalian pathogenicity given the body temperature of these animals, including man. Similarly, a microbial biopesticide labeled for use at residential sites only is unlikely to result in significant exposure to marine and estuarine environments. Explanation of factors affecting the applicability of a study outcome to a risk determination may be used to satisfy some data requirements. As always, it is important to discuss this with regulators prior to conducting any studies.

Relative to a BPN or an EUP, the number of studies requiring empirical data generation applied to the issuance of a Section 3 registration are typically greater as this regulatory action often coincides with commercial use on a larger scale than either of the two preceding regulatory actions. For both BPN and EUP actions, the

scope of the exposure of man and the environment to the novel pesticide is significantly reduced as compared to commercial use in most instances. Hence, the data and information required for an EUP or small scale field test under a BPN are more often limited to concerns of human health (e.g., infectivity) and environmental persistence than with longer term non-target effects, which will be addressed at the time of registration, with data obtained through earlier field tests in most cases.

It should be noted that all pesticide registrations are subject to periodic review and re-registration procedures as FIFRA is a licensing statute and statutory requirements exist in order to maintain that license or registration in good standing in order to enter the product into commerce.

The first genetically engineered MPCA registered under FIFRA was a pair of *Pseudomonas fluorescens* strains, each modified with a different type of δ-endotoxin from *B. thuringiensis,* for insect control. Mycogen chose to express their *kurstaki* and *san diego* type endotoxins in *Ps. fluorescens* to provide for adequate expression and accumulation of protein toxin, but also as a means of reducing inactivation of these proteins by ultraviolet light. These products were referred to as MVP and M-Trak, respectively, and did not contain any live organisms, so the risk assessment was not concerned with pathogenicity or infectivity issues.

4.3 Risk Assessment Considerations

4.3.1 FIFRA

As noted above, FIFRA's standard for registration decisions involves an assessment of risks and benefits of using a pesticide. This is to include a biological analysis of potential effects upon man and the environment as well as social and economic considerations resulting from a regulatory decision. The inclusion of an explicit risk-benefit calculation distinguishes FIFRA from most other U.S. environmental statutes.

One of the primary benefits of a biopesticide is the replacement of control measures that may pose greater risks, such as groundwater contamination, toxicity to non-target organisms, or dietary risks to infants and children. To date, decisions to approve nuclear polyhedrosis viruses (NPVs), plant viruses and bacteriophage have relied primarily on their lack of toxicity to all organisms except target pests with little or no animal testing conducted. EPA considers possible benefits that might result from use of viruses such as the NPV AcMNPV/LqhIT2 (OSTP 2001). Application of AcMNPV/LqhIT2 would likely reduce the use of other insecticides and thereby would avoid the types of impacts those less specific insecticides might have had, if applied to the same acreage as AcMNPV/LqhIT2.

Targeting an insect-specific toxin to the 'point of feeding' of pest insects should minimize the impact on non-target organisms and minimize ground water contamination, as may occur with use of more environmentally persistent chemical pesticides. Because many of the previously deployed insecticides were broad-spectrum in their activities, the potential for impacts on the beneficial insect populations was significant.

Populations of beneficial insects should increase over time as more MPCAs with host specificity are used and fewer broad-spectrum pesticides are applied. This has been shown in the context of B.t. corn crops, where increased abundance of arthropods was noted in B.t crop fields when compared to conventionally bred maize treated with insecticides (Marvier et al. 2007). Since some insecticides have effects on non-insect organisms (e.g. earthworms, nematodes), the reduction or elimination of these broad-spectrum pesticides will help to nurture these populations as long as cultural practices of soil management are adequate.

Additionally, the exposure of farm workers, pesticide applicators and the public at-large is often reduced when a biological pesticide takes the place of a chemical spray alternative. For example, residues on food are less of a concern with AcMNPV/LqhIT2, because the insect neurotoxin is known to be non-toxic to humans and other mammals. Spray drift is often problematic with chemical applications, but this is not a significant issue with target specific NPVs.

FIFRA also requires special consideration of public health pests, such as disease vectoring mosquitoes, cockroaches and rodents. Data detailing the ability of the MPCA to manage a pest situation are required for all registrations, however, these data must be submitted and reviewed for those involving public health pests prior to any such regulatory action being considered.

4.3.2 FFDCA

The Federal Food, Drug, and Cosmetic Act is largely the purview of the US Food and Drug Administration, except for residues of pesticides that may occur in food and feed (Section 408, FFDCA). Microbial biopesticides that pass Tier I testing without evidence of toxicity or pathogenicity will most often qualify for an exemption from the requirement of a numerical food tolerance (also referred to as a Maximum Residue Level in some countries). This regulatory action, determined following risk assessment and literature review, has afforded the determination that any level of the microbe present in food and feed resulting from use of the product as specified on the FIFRA label, will not result in harm by a variety of exposures. Among the exposure scenarios assessed for food safety are ingestion through food or water, inhalation, dermal and eye contact, and injection. While effects may be evident in some of these tests, the probability of exposure is also a consideration. Specific areas addressed under FFDCA (as applicable to microbial pesticides) are acute, subchronic and chronic dietary risks, occupational exposures, drinking water exposures, effects to the immune and endocrine systems, any dose response related information, exposures associated with day cares, residences and schools, exposure of sensitive populations, such as infants or children, aggregate effects for multiple exposures, and cumulative effects.

When assessing MPCA, there are the three endpoints of concern: infectivity, pathogenicity and toxicity. In some cases an analysis of potentially toxic metabolites is included in the food safety risk assessment and review of the primary literature. Some microbial species are known to produce metabolites or toxins which can have adverse effects upon man and livestock following consumption.

Note, if an organism is not completely identified or is closely related to a human pathogen, i.e., in the same genus, the literature review and subsequent risk assessment should be broad enough to cover the eventuality that the relevant pathogenicity factors and/or toxins are ruled out as not present in the test strains proposed for use as a pesticide.

4.4 Entomopathogenic Nematodes

Entomopathogenic nematodes have been applied to pest management of insects in diverse agricultural settings (de Doucet et al. 1998; Head et al. 2000; Martin 1997). While the number of nematode genera infecting insects and other arthropods is large and diverse, most of the research and development interest has been with the Steinernematid and Heterorhabditid groups targeting agricultural insect pests (Grewal and Peters 2005). Both of these genera rely on symbiotic (phoretic) bacteria to effect a lethal septicemia upon their hosts which results in degradation of internal tissues and organs, death of the insect host, and reproduction of the nematode and symbionts.

Members of the genera *Steinernema* and *Heterorhabditis* differ in their strategies of host location, host specificity, and survival mechanisms, they are both inherently susceptible to heat and desiccation in the soil environment. As a means of enhancing the heat tolerance of *Heterorhabditis bacteriophora*, an hsp70A gene from *Caenorhabditis elegans* was introduced to juvenile nematodes (Hashmi et al. 1995; Wilson et al. 1999). Although this effort was ultimately not successful at the field level in providing the necessary level of heat tolerance, it nonetheless raised some interesting regulatory issues (Gaugler et al. 1997).

The Code of Federal Regulations defines microorganisms considered as biopesticides to include viruses, bacteria, protozoa, algae and fungi (FR 2007). Absent from this list are nematodes and certain other microscopic, multicellular invertebrates. Nematodes may be included as biocontrol agents subject to oversight under the Plant Pest Act, yet this is less than apparent.

According to the Coordinated Framework for Biotechnology (OSTP 1986) when referring to EPA's oversight, "The Agency has determined that certain non-microbial organisms which fall within the definition of biological control agents are already addressed by other agencies, specifically USDA and the Department of the Interior. Examples of these biological control agents are vertebrates, insect predators, nematodes, and macroscopic parasites. Therefore, pursuant to section 25(b) of FIFRA and 40 CFR 162.5(c)(4), these nonmicrobial biological control agents have been exempted from regulation under FIFRA. However, if EPA, in cooperation with other agencies, determines that certain biological control agents exempted by § 162.5(c)(4) are not being adequately regulated, these organisms will be referred to the attention of the appropriate agency or added to the exceptions in § 162.5(c)(4) by amendment. In the latter case, those organisms would no longer be considered exempt from the provisions of FIFRA."

While entomopathogenic nematodes are included in this exemption, genetic engineering of either the nematode or the microbial symbiont could bring the new product back under FIFRA oversight as a pesticide.

Genetic engineering of the microbial symbionts (i.e., *Xenorhabdus* spp.; *Photorhabdus* spp.) would bring these organisms under the regulatory umbrella of the USDA-APHIS and EPA, however, modification of the nematode itself does not meet existing regulatory thresholds (FR 2007; Gaugler et al. 1997; Gaugler, personal communication). It should be noted that in the U.S., the importation and interstate movement of exotic entomopathogenic nematodes may be regulated by the USDA-APHIS' Plant Protection and Quarantine group (Rizvi et al. 1996; Selçuk et al. 2003) under the Plant Protection Act of 2000.

During laboratory and growth chamber experimentation with the *H. bacteriophora* hsp70A transformants, this issue was raised to the USDA-APHIS and EPA-BPPD for clarification (Randy Gaugler, personal communication; Chris Wozniak, personal communication). At the time, neither agency indicated jurisdictional oversight of these GE nematodes, but suggested that the Center for Disease Control be contacted as well. Communication with CDC (Wozniak, personal communication) likewise indicated that they did not claim oversight of the organisms for the intended purpose (i.e., pest control).

Faced with this lack of Federal oversight, yet concerned with public perception and local (i.e., State, University Institutional Biosafety Committees) considerations, the lead investigator, Dr. Randy Gaugler of Rutgers University, requested a review of the *H. bacteriophora hsp*70A, as applied to insect pest management, by the USDA-APHIS. This review resulted in a finding of no significant impact (FONSI) by the agency and a determination that environmental release would not result in injury to agricultural plants or their products as determined under the Plant Pest Act. Note that this finding does not preclude potential regulatory action by State or other local authorities, as is the case with all microorganisms, including pesticidal agents, intended for release into the environment.

While the lack of Federal regulation has obviously reduced costs and time necessary to bring an entomopathogenic nematode product to market, some have opined that this lack of oversight has resulted in some inferior products with exaggerated claims (Weinzierl et al. 2005). At least one of the authors (CAW) has had this unfortunate experience!

4.5 Considerations of Genetic Engineering and Gene Transfer

4.5.1 Public Perception of GE Microbials

During the early stages of the development of GE microorganisms, significant public debate occurred regarding the human health and environmental safety of these novel products of biotechnology (Marchant 1988; Barinaga 2000). As is often the case with public reaction to new technologies, the debate was not always centered on scientific facts or reasoned discussion, but was taken up by opponents of biotechnology as a

crusade against development of genetically engineered organisms regardless of intent or merit. Additionally, debate within the scientific community was needed to develop a regulatory system capable of responding to novel products and nuances to the technology as they developed. As evidenced by the early field experiments with ice-minus bacteria for frost prevention on strawberries and potatoes (Crawford 1986; Marchant 1988; Barinaga 2000), or the intentional degradation of an oil spill by hydrocarbon munching pseudomonads (Van 1989), public and, therefore, political considerations have influenced the field release and commercialization of GE microbes. Others have also expressed concerns (Dixon 2008).

Consideration of public perception and understanding of this novel technology led to business decisions that apparently did not necessarily reflect the actual science or potential risk associated with the proposed release of a particular GE microbial pest control agent. As is the case with GE plants, commercial considerations and the threat of lawsuits, with or without merit, persuaded individual concerns to halt research and development programs that may have lead to more environmentally benign alternative pest management measures (Phil Hutton Personal communication). Although regulatory requirements by EPA and USDA-APHIS may result in greater costs and longer lead times for commercialization of GE microbial products, we believe that, at least in some cases, companies were seeking regulatory approval as a means of indicating the safety of these products and did not perceive regulatory requirement as a deterrent to application of the products to market (Wrubel et al. 1997). Given the furor over the ice-minus and concurrent microbial field tests, regulatory oversight and approval may have enhanced public acceptance.

Many years later, as genetic engineering technology has progressed, significant numbers of GE microbial pest control agents exist on the market without the fanfare and protests characteristic of the early years of this technology. We believe that this bodes well for the potential of this technology to reduce the application of less environmentally benign technologies that ultimately have the potential for greater environmental effects.

4.5.2 Future for GE Microbials in Pest Management

The field of agricultural biotechnology has grown and developed so rapidly in the last 20 or so years that avenues to be taken, which we had not even anticipated 5 or 10 years ago, will continue to astound us in the future. The majority of this activity, at least in traditional agricultural terms, has been directly through engineering of plants for a variety of purposes, while the application of rDNA technology to microbial agents for pest and disease control has been slow in comparison. As can be evidenced by Table 4.1, the number of research efforts aimed at pest control through genetic engineering of MPCA have been numerous over the years. But, these efforts appear to have slowed, as recent actions are relatively few. There is, however, reason to expect that this may change in the future, at least in US and Canadian applications.

Despite the fact that some individuals are uncomfortable with microbes in general, based largely on a lack of understanding and encouraged by germ phobias, the instances where genetically engineered microbials have been utilized for nitrogen

fixation, soil amendments, biological control, and in bioremediation have not garnered the negative publicity to the degree that GE crop plants developed for agronomic, quality trait, and pest control purposes have. This was clearly not the case early on with the advent of biotechnology in agriculture – as was demonstrated by the furor over the early ice-minus field trials with pseudomonads in California or the first release of oil-degrading bacteria for cleanup of petroleum spills in marine environments.

The lack of attention to GE MPCA and other microbials may be in part due to the continued rancor over GE crops. There is also a common thread of mistrust among some of these groups toward large corporate interests (i.e., seed companies) such that the continued research and application of GE microbes flies largely under the radar of those who claim an innate aversion to this most promising of modern technologies. The majority of GE MPCAs are developed by small to mid-size companies without the visibility of those heavily involved in crop biotechnology.

One must also consider the use of GE microbes in food processing (e.g., chymosin, ascorbic acid production, flavor enhancers), even in countries where biotechnology is publically and officially shunned by many (e.g. the EU; GMO Compass 2010). These organisms and their products, when used as food processing aids, fail to trip the regulatory requirement for food labeling in stark contrast to those food and feed products derived from products of crop biotechnology. Perhaps this level of familiarity has garnered some trust with consumers or it simply has not made news enough to be noticed. Either way, it could bode well for GE microbial agents applied to agriculture and the environment.

4.6 TSCA Risk Assessment of Intergeneric Microorganisms

4.6.1 TSCA Regulation of Microorganisms

The United States Environmental Protection Agency is responsible for reviewing the risks associated with the commercial use or importation of chemical substances, including certain genetically modified microorganisms, under Section 5 of the Toxic Substances Control Act (TSCA). TSCA specifically excludes from review certain products that are subject to review by other federal agencies or under other statutes, including tobacco, nuclear materials, pharmaceuticals and cosmetics, and pesticides (but not pesticidal intermediates). TSCA's regulation of microorganisms is limited to those microorganisms that are "new", meaning that they are not listed on the TSCA Inventory of Chemical Substances. In this context, "new" microorganisms have been defined as those that are intergeneric, meaning that there has been the deliberate combination of genetic material originally isolated from organisms classified in different taxonomic genera. Also included in the definition of an intergeneric microorganism is a microorganism constructed with synthetic genes that are not identical to DNA that would be derived from the same genus as the recipient microorganism. Exclusions from TSCA review include naturally occurring microorganisms, as they

are considered to be implicitly listed on the TSCA Inventory, genetically engineered microorganisms other than intergeneric (e.g., intrageneric, physical or chemically mutagenized microorganisms), and intergeneric microorganisms resulting only from the addition of well-characterized, non-coding regulatory regions. TSCA section 5 only applies to microorganisms that are manufactured, imported, or processed for commercial purposes.

Intergeneric microorganisms subject to review under TSCA include a wide variety of biotechnological applications since TSCA is a gap-filling statute for biotechnology products not regulated under other statutes. Intergeneric microorganisms that may be subject to review under the Biotechnology Rule (40 CFR Parts 700,720, 721, 723, and 725 Microbial Products of Biotechnology: Final Regulation Under the Toxic Substances Control Act, FR Vol 62 No. 70 17909–17958, April 11, 1997) may be in applications including but not limited to biofuel production, biomass conversion, waste treatment, bioremediation, biomining, mineral leaching, oil recovery, desulfurization of fossil fuels, biofertilizers, biosensors, closed system fermentation for the production of enzymes and specialty chemicals, and pesticidal intermediates. Among these, biofertilizers (e.g., nitrogen fixers, mycorrhizae, phosphate solubilizers, etc.), algal biofuels, pesticidal intermediates, and perhaps, biosensors could have agricultural uses.

4.6.2 Categories of Premanufacturing Oversight

4.6.2.1 Microbial Commercial Activity Notice (MCAN)

Prior to manufacture or importation of an intergeneric microorganism, companies must make an appropriate submission to EPA's Office of Pollution Prevention and Toxics (OPPT). Subpart D of part 725 of the Biotechnology Rule establishes the reporting program for new microorganisms. New microorganisms that are to be manufactured or imported for distribution into commerce requires the submission of a Microbial Commercial Activity Notice (MCAN) 90 days prior to initiating manufacture or import, unless the activity is eligible for one of the specific exemptions.

The purpose of the MCAN is to supply EPA with information necessary to identify and list the new microorganism on the TSCA Inventory of Chemical Substances, and to determine whether the microorganism and the associated manufacture or importation may present an unreasonable risk of injury to human health or the environment. The MCAN information requirements closely parallel those developed for traditional chemical Premanufacturing Notices and differ only to the extent necessary to accommodate the specific characteristics of living microorganisms versus chemicals. All information on the microorganism identity and data on its actual and potential effects on human health and the environment that are available to the submitter, or are reasonably ascertainable are required in the MCAN. A detailed description of the genetic modifications to the recipient microorganism is necessary, along with data on the stability of inserted genetic material in the production strain

and the potential for transfer of this material to other organisms in the environment. In addition, a detailed complete description of the manufacturing process and design, production volumes, and containment and inactivation procedures are required. The requirements for information to be included in the MCAN are codified at § 725.155 and § 725.160.

4.6.2.2 Exemptions from Full Premanufacturing Notification

Research and Development Exemption

One exemption from MCAN reporting is the R&D Exemption. This is a complete exemption from TSCA § 5 reporting for certain R&D activities that are (1) conducted in contained structures, and (2) are subject to regulation by another Federal agency. As discussed in Subpart E of the Biotechnology Rule and codified at § 725.232, activities that meet these criteria are exempt from EPA review, reporting, and record keeping requirements for contained research conducted by researchers who are required to comply with the NIH Guidelines for Research Involving Recombinant DNA Molecules (http://oba.od.nih.gov/rdna/nih_guidelines_oba.html).

Other manufacturers conducting contained TSCA research and development activities that are not subject to regulation by the NIH Guidelines may qualify for a more limited contained R&D exemption under § 725.234 and § 725.235. This exemption for R&D in contained structures specifies factors that a technically qualified individual (TQI) must consider in selecting the appropriate containment for this exemption. A structure is defined as a building or vessel which effectively surrounds and encloses the microorganism and includes features designed to restrict the microorganism from leaving. In proposing the Biotech Rule, EPA envisioned that this exemption would most likely apply to research performed in contained structures such as buildings, including laboratories, greenhouses, and pilot fermentation plants. etc., and in certain bioreactors used for waste treatment. However, other forms of structures could be used. EPA's approach relies on the experience and judgment of the TQI, recognizing that many different kinds of microorganisms displaying a wide range of characteristics could potentially be used in research. It also recognizes that appropriate types of controls (e.g., procedural. mechanical. and/or engineering) will vary with the microorganism and type of research. EPA expects that the TQI will be cognizant of these factors when selecting containment and inactivation controls appropriate to the microorganism(s) being utilized. The technically qualified individual is required to keep records to document both compliance with the containment requirements and compliance with the notification process for employees involved in the R&D process.

A major consideration of the R&D exemption in a contained structure is the structure itself. EPA may interpret the definition of a structure broadly given the intention of freely permitting research with contained microorganisms that meet the criteria of § 725.234. However, EPA encourages potential researchers who wish to perform their research in atypical contained structures to confer with EPA prior to initiating

their effort to confirm that the structure is considered "contained". There may be instances in which a TSCA Environmental Release Application (TERA), which is a submission for field testing or intentional environmental release, may be required if the structure is not deemed "contained" (see below for TERA requirements).

Tier I and Tier II Exemptions

There are exemptions from MCAN reporting for certain industrial microorganisms used in closed systems so they likely have limited, if any, relevance to typical agricultural applications. As described in Subpart G, these Tier I and Tier II exemptions for closed systems are based on a three-pronged approach: use of a microorganism with a history of safe use, criteria that ensure the safety of the introduced DNA, and conditions for containment and inactivation of the microorganism to ensure low releases from the manufacturing/production facility. To qualify for the Tier I exemption, a manufacturer must use one of the ten recipient organisms listed at § 725.420 that have undergone categorical risk assessment, or any such microorganism subsequently listed after promulgation of the Biotechnology Rule through a petition process described in § 725.67. Currently, the eligible recipient microorganisms include the five bacteria *Acetobacter aceti, Bacillus licheniformis, Bacillus subtilis, Clostridium acetobutylicum, Escherichia coli* K-12, and the five fungi *Aspergillus niger, A. oryzae, Penicillium roqueforti, Sacharromyces cerevisiae,* and *S. uvarum.* In addition to the use of an approved recipient microorganism, there are four criteria for the genetic material introduced into these strains. There are also specific criteria for releases from the manufacturing facility and for inactivation of liquid and solid waste streams. For those manufacturers meeting Tier I requirements, only a brief notification to the Agency stating that fact is necessary. A manufacturer, who meets only the first two conditions of the Tier I exemption, but not the containment and inactivation criteria must submit a Tier II exemption notice to the Agency for a review of the process design and containment/inactivation conditions appropriate for the intergeneric microorganism.

Test Marketing Exemption (TME)

Another exemption from MCAN reporting requirements is the Test Marketing Exemption (TME) noted at § 725.300. Test marketing activities usually involve limited sale or distribution of a substance within a predetermined period of time to determine its competitive value when its market is uncertain. In general, EPA suggests that manufacturers who intend to test market a new microorganism file a MCAN rather than request a Test Marketing Exemption. However, there may be situations in which this exemption is appropriate, such as for microorganisms which were previously reviewed by EPA at the R&D stage. In addition to the general administrative requirements, certain technical information is required for each TME submission as noted in § 725.350 and § 725.355.

4.6.2.3 TSCA Experimental Release Application (TERA)

Another exemption from MCAN reporting requirements is available for R&D activities. The TSCA Experimental Release Application, described in Subpart E at § 725.238, is an exemption for R&D involving an intentional environmental release of an intergeneric microorganism. This exemption is likely to be a common one for many agricultural uses (e.g., biofertilizers, algae for biofuel production), as they generally involve field tests or may involve some release of subject microorganisms. Also, as previously mentioned, a TERA may be necessary for some contained R&D activities if such R&D is conducted in an atypical structure that does not meet the regulatory definition of a contained structure. The TERA is essentially an abbreviated MCAN for a field test or other intentional environmental introduction with a shortened review period of 60 days, although EPA may extend the review period for good cause. EPA must approve the TERA, with or without conditions, before the researcher may proceed, even if the 60-day period expires. EPA's approval is limited to the conditions outlined in the TERA notice and approval for the specific field test at the specified site(s).

A TERA must contain all available data in the possession or control of the submitter or reasonably ascertainable by the submitter on the microorganism(s) and the research and development activities that will allow EPA to make a reasoned evaluation of the planned test in the environment. The TERA must contain microorganism identity information and all available data concerning actual or potential effects on health or the environment of the new microorganism along with the phenotypic and ecological characteristics of the microorganism as they relate directly to the conditions of the proposed R&D activity. Persons applying for a TERA must also submit information about the proposed field testing activity including the objectives and significance of the activity with a rationale for testing in the environment, the numbers and frequency of microorganisms released by the proposed application method(s), the presence of target organisms, if applicable, and a full characterization of the test site(s) including location, geographical, physical, chemical, and biological features, and proximity to human habitation or activity. Also needed is a description of confinement procedures, mitigation and emergency procedures, and procedures for routine termination of the activity. The exact information requirements for a TERA are codified at § 725.255 and § 725.260.

Exemptions from a TERA for Eligible Microorganisms

There is an exemption from TERA reporting requirements for R&D field testing of two microorganisms with which EPA has had sufficient experience to determine that a submission is no longer needed. The exemption applies to two eligible microorganisms, *Bradyrhizobium japonicum* and *Sinorhizobium* (formerly *Rhizobium*) *meliloti*) providing certain conditions of the microorganisms and of the field testing are met. The introduced genetic material must comply with certain restrictions, the field testing must occur on no more than 10 terrestrial acres, and appropriate

containment measures must be selected to limit dissemination (see § 725.238 and § 725.239).

This TERA Exemption requires no upfront reporting to EPA, although a certification statement and recordkeeping are required. Guidance on how to submit a certification statement to EPA and on the recordkeeping requirements for field tests with these bacteria is provided at § 725.238.

4.6.3 Risk Assessment Process

Within the specified time period for each type of submission, EPA staff conduct a risk assessment on the intergeneric microorganism under the paradigm that Risk = Hazard × Exposure. There are a number of separate assessments made that are integrated into a final risk assessment. The components of the risk assessment include (1) a verification of the identification of the subject microorganism, (2) a human health hazard assessment, (3) an ecological effects hazard assessment, (4) a report that analyzes the construction of the microorganism and summarizes the pertinent chemical information and production volume known as the chemistry report, (5) an analysis of the genetic construct that evaluates any potential hazards associated with the genetic modifications and the potential for horizontal gene transfer, (6) an engineering report that assesses worker exposure and microbial releases to the environment through manufacturing or during field applications, and (7) an exposure assessment that evaluates the potential for survival, reproduction, and dissemination of the microorganism, and the exposure of the microorganism to environmental receptors and to the general population.

As noted below, there is no provision for a specified schedule of information elements under TSCA. Rather submitters must provide to EPA all relevant data and information in their possession or reasonably ascertainable. These data must be sufficient to enable EPA to complete a risk assessment. If a submission of any type contains insufficient information to proceed with a review, EPA may request an extension from the submitter to allow the submitter to provide the necessary information. EPA also has risk management options that may be employed to mitigate the effect of uncertainty due to data or information limitations as described below.

Since TSCA is a risk-benefit statute, the risks of using the microorganism determined in the risk assessment are weighed against the benefits to society (that are evaluated in an economics analysis) to arrive at the final risk management decision. Possible outcomes of the review process include a determination that there is (1) sufficient information to determine that the microorganism presents "no unreasonable risk of injury to human health or the environment" in which case the Agency takes no regulatory action and the company may commence manufacture after 90 days, (2) sufficient information to determine that the microorganism presents "an unreasonable risk of injury to human health or the environment" which means the Agency would take regulatory action to prohibit or restrict the production or use of the microorganism, and (3) insufficient information to determine effects, but the

possibility exists for unreasonable risk and/or substantial/significant exposure, in which case the Agency may negotiate a Section 5(e) Consent Order to restrict the use, and to specify the data needed to lift the Consent Order. The key element to the possible outcomes of EPA's review process is the amount of information that the Agency is supplied with or can obtain concerning the microorganism in order to make a determination of whether or not the use of the microorganism presents an unacceptable risk of injury to human health or the environment.

4.6.4 Data and Information Needs

Unlike many other statutes under which biotechnology products are reviewed, TSCA does not have specific initial data requirements. Rather, the submitter is required to provide relevant data and information that are available or reasonably ascertainable with the notification to EPA. In contrast, with microbial pest control agents (MPCAs) which are reviewed by EPA's Office of Pesticide Programs (OPP) under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), there are a number of specific pathogenicity/toxicity/infectivity tests that must be conducted and submitted to the Agency. MPCAs, by their very nature, are designed to be either pathogenic or toxic to some pest, and consequently, their effects of pathogenicity/toxicity are fairly straightforward. The microorganisms that fall under TSCA review differ in that most are not likely pathogenic or toxic, but primarily are benign recipient microorganisms genetically engineered to synthesize a particular product or accomplish a particular task or transformation.

Obtaining sufficient information about the submission microorganism from the manufacturer or importer so that a scientifically credible risk assessment can be conducted by the Agency is critical to the review process. Information needs match the set of individual assessments (e.g., human health, ecological effects, etc.) that go into the comprehensive risk assessment described previously. Since combinations of microorganism and proposed use can vary widely, EPA prepared a guidance document, "Points to Consider in the Preparation of TSCA Biotechnology Submissions for Microorganisms, June 2, 1997" (hereafter referred to as the Points to Consider document). This document is intended to assist manufacturers or importers in providing EPA's Office of Pollution Prevention and Toxics with both appropriate and sufficient information for EPA to conduct a robust risk assessment. It is intended that the Points to Consider document be a "living document" in that it will be updated periodically to reflect state-of-the-art biotechnological applications, risk assessment methodology, and current knowledge of microbial processes and characterization.

Although there are no data requirements that are applied routinely to each case, information that is both accurate and sufficient is necessary to evaluate the risks posed by the manufacture and use of genetically modified microorganisms. Each submitter must supply, as part of its notification requirements, all relevant data and information in its possession, or that is otherwise reasonably ascertainable. Information available in the literature or from sources other than the submitter is also used by the Agency in the evaluation of the hazards posed by the microorganism

and its ability to survive in the environment. The effects of genetic modifications of the recipient microorganism are then evaluated. For instance, if a recipient bacterium is known from the literature not to be a frank human pathogen, then it is unlikely that the introduction of one or several genes will create a pathogenic microorganism *de novo*. Likewise, if from the literature it is known that the recipient microorganism survives well in the environment, then the intergeneric microorganism also might be expected to survive well depending on whether the genetic modification altered any genes key to its survival characteristics. The Points to Consider document has been provided to guide submitters in selecting all the relevant information that the Agency may need for the review of all possible types of microorganisms and applications that may be subject to review under TSCA. All of the points or issues in the guidance document may not be appropriate for all cases. This document is not a schedule of data requirements but rather essentially a menu of data elements from which submitters are expected to choose the ones relevant to their particular microorganism and application. For example, information on substrate range and metabolic pathways may be applicable for a microorganism designed for bioremediation, but would be irrelevant for a microorganism designed for symbiotic nitrogen fixation. Identification of possible nontargets, i.e., potential legume hosts, may be important for symbiotic nitrogen-fixing rhizobia, but irrelevant to a microorganism used in a closed system for making an algal biofuel.

4.6.5 Applications of Genetically Engineered Microorganisms Reviewed to Date

4.6.5.1 Past Applications

A wide variety of intergeneric microorganisms have been reviewed under TSCA since the mid 1980s. Prior to the promulgation of the Biotechnology Rule in 1997, intergeneric microorganisms with TSCA uses were reviewed on a voluntary basis under the chemical Pre-Manufacturing Notification (PMN) system. Those intergeneric microorganisms and their genetic modifications with relevance to agriculture are listed in Table 4.2.

Following the promulgation of the Microbial Biotechnology Rule, various submissions types discussed above for intergeneric microorganisms have been received by the Agency. The majority of the submissions reviewed by EPA since publication of the Biotechnology Rule have been for closed system fermentation for enzyme production which were not relevant to agriculture, and thus, will not be elaborated on here. A complete list of all intergeneric microorganisms reviewed under TSCA to date can be obtained on the Biotechnology Program's website (http://www.epa.gov/oppt/biotech) under Notifications.

Table 4.3 presents those intergeneric microorganisms reviewed by EPA under TSCA since the promulgation of the Biotechnology Rule, having relevance to agriculture, all of which were TERA submissions.

Table 4.2 Agriculturally relevant genetically engineered microorganisms reviewed by EPA under TSCA under voluntary PMNs

Fiscal year	Company	Recipient microorganisms	Introduced genetic material	Purpose
1987	BioTechnica International, Inc.	Three strains of *Rhizobium meliloti* (*Sinorhizobium meliloti*)	Nitrogen fixation genes and antibiotic resistance genes	Symbiotic nitrogen fixation in alfalfa
1987	Monsanto Agricultural Company	*Pseudomonas aureofaciens* (currently *Pseudomonas chlororaphis*)	*lac* genes from *Escherichia coli*	"Marker" genes for monitoring the microorganism in the field
1988	BioTechnica, Agriculture, Inc.	Eight strains of *Rhizobium meliloti* (*Sinorhizobium meliloti*)	Nitrogen fixation genes and antibiotic resistance genes	Symbiotic nitrogen fixation in alfalfa
1988	BioTechnica, Agriculture, Inc.	Four strains of *Rhizobium meliloti* (*Sinorhizobium meliloti*)	Nitrogen fixation genes and antibiotic resistance genes	Symbiotic nitrogen fixation in alfalfa
1989	BioTechnica, Agriculture, Inc.	One strain of *Rhizobium meliloti* (*Sinorhizobium meliloti*)	Nitrogen fixation genes and antibiotic resistance genes	Symbiotic nitrogen fixation in alfalfa
1989	BioTechnica, Agriculture, Inc.	Two strains of *Bradyrhizobium japonicum*	Nitrogen fixation genes and antibiotic resistance genes	Symbiotic nitrogen fixation in soybeans
1990	BioTechnica, Agriculture, Inc.	One strain of *Rhizobium meliloti* (*Sinorhizobium meliloti*)	Nitrogen fixation genes and antibiotic resistance genes	Symbiotic nitrogen fixation in alfalfa
1991	Mycogen Corporation	Two strains of *Pseudomonas fluorescens*	Delta endotoxin genes from *Bacillus thuringiensis* var. *kurstaki* or var. *san diego*	TMEs of pesticidal intermediates of pesticides consisting of encapsulated killed cells for control of beetles and caterpillar pests
1992	Mycogen Corporation	Sixteen strains of *Pseudomonas fluorescens*	Delta endotoxin genes from *Bacillus thuringiensis* var. *kurstaki* or var. *san diego*	TMEs of pesticidal intermediates of pesticides consisting of encapsulated killed cells for control of beetles and caterpillar pests
1992	Research Seeds, Inc. (purchaser of BioTechnica Agriculture, Inc. strains)	Five strains of *Rhizobium meliloti* (*Sinorhizobium meliloti*)	Nitrogen fixation genes and antibiotic resistance genes	Symbiotic nitrogen fixation in alfalfa

1993	Research Seeds, Inc.	Five strains of *Rhizobium meliloti* (*Sinorhizobium meliloti*)	Nitrogen fixation genes and antibiotic resistance genes	Test marketing large scale field trials for one strain, RMBPC-2, symbiotic nitrogen fixation in alfalfa
1994	Research Seeds, Inc.	Four strains of *Bradyrhizobium japonicum*	Nitrogen fixation genes and antibiotic resistance genes	Symbiotic nitrogen fixation in soybeans
1994	Research Seeds, Inc.	Five strains of *Rhizobium meliloti* (*Sinorhizobium meliloti*)	Nitrogen fixation genes and antibiotic resistance genes	Additional test marketing field trials for one strain, RMBPC-2, symbiotic nitrogen fixation in alfalfa
1995	Research Seeds, Inc.	Five strains of *Rhizobium meliloti* (*Sinorhizobium meliloti*)	Nitrogen fixation genes and antibiotic resistance genes	Additional test marketing field trials for one strain, RMBPC-2, symbiotic nitrogen fixation in alfalfa
1995	Univ. of Wisconsin, USEPA Office of Research & Development	Two strains of *Rhizobium meliloti* (*Sinorhizobium meliloti*)	Nitrogen fixation genes and antibiotic resistance genes	Symbiotic nitrogen fixation in transgenic alfalfa
1995	Mycogen Corporation	One strain of *Pseudomonas fluorescens*	Delta endotoxin genes from *Bacillus thuringiensis*	TMEs of pesticidal intermediates of pesticides consisting of encapsulated killed cells for control of lepidopteran pests

Table 4.3 Agriculturally relevant genetically engineered microorganisms reviewed by EPA under TSCA under the biotechnology rule as TERAs

Fiscal year	Recipient microorganism	Introduced genetic material	Purpose
1998	Three strains of *Bradyrhizobium japonicum*	Nitrogen fixation and nodulation genes	Nitrogen fixation in soybeans
1999	Three strains of *Bradyrhizobium japonicum*	Nitrogen fixation and nodulation genes	Nitrogen fixation in soybeans
2003	*Alcaligenes xylosoxidans* subspecies *denitrificans* strain AL6.1	DsRed fluorescent protein marker gene	Detection in the environment – for eventual insertion of pesticidal gene for control of *Xylella fastidiosa* (Pierce's disease of grapes)
2004	*Alcaligenes xylosoxidans* subspecies *denitrificans* strain AL6.1	DsRed fluorescent protein marker gene	Detection in the environment – for eventual insertion of pesticidal gene for control of *Xylella fastidiosa* (Pierce's disease of grapes)
2005	*Alcaligenes xylosoxidans* subspecies *denitrificans* strain AL6.1	DsRed fluorescent protein marker gene	Detection in the environment – for eventual insertion of pesticidal gene for control of *Xylella fastidiosa* (Pierce's disease of grapes)

4.6.5.2 Potential Future Applications

Biofertilizers

As previously mentioned, there are many biotechnology applications of genetically engineered microorganisms that potentially may fall under the purview of TSCA including a number of uses that are relevant to agriculture. These include intergeneric microorganisms used as biofertilizers such as symbiotic nitrogen-fixers such as *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*. Field tests of numerous intergeneric rhizobia have gone through review under TSCA, and one particular strain of *S. meliloti,* RMBPC-2, was approved in 1997 for limited commercialization. In the future, there could be more submissions for more rhizobia for increased nitrogen-fixation ability, or perhaps, for enhanced nodulation efficiency. In addition, applications for other symbiotic nitrogen fixers, such as the actinomycete *Frankia* which is a Gram positive bacterium that forms symbiotic relationships with certain plants such as woody angiosperms referred to as actinorhizal plants, are a possibility. There may also be submissions for free-living nitrogen fixing microorganisms. In addition to nitrogen-fixing intergeneric microorganisms, other biofertilizer applications that would be reviewed under TSCA include phosphate-solubilizing microorganisms, mycorrhizal fungi, or other endophytic microorganisms that aid in nutrient absorption, plant hormone production, or other mechanisms that may increase plant productivity.

Biosensors

Microbial biosensors consist of the use of a microorganism that has some sort of reporter molecule that indicates the presence of a target molecule. The reporter genes used in recombinant DNA technology for microbial biosensors include those that can result in a signal that can be visible to the naked eye such as color production (e.g., blue color resulting from the breakdown of X-galactopyranoside by β-galactosidase), bioluminescence (e.g., *luc* or *lux* genes), or fluorescence (e.g. *gfp* or DsRed). One of the earliest genetically engineered microorganisms to be field tested was Monsanto's *Pseudomonas chlororaphis* (formerly *P. aureofaciens*) into which the β-galactosidase gene was inserted to enable detection of the microorganism in the environment. The *A. xylosoxidans* reviewed under TSCA that was eventually to be manipulated with pesticidal genes contained the DsRed protein for detection the microorganism in the environment as well. Other biosensors with reporter genes for detection of particular target molecules have been reviewed under TSCA as well. One such biosensor was a strain of *Pseudomonas fluorescens* Hk44 containing *lux* bioluminescence genes for detection of polycyclic aromatic hydrocarbons including naphthalene and methyl salicylate. Another reporter biosensor was a strain of *Pseudomonas putida* with genes for detection of unexploded ordinance, specifically trinitrotoluene (TNT). Another biosensor microorganism, a *P. putida* containing *lux* genes was reviewed that was developed for detection of trichloroethylene (TCE) and BTEX compounds (benzene, toluene, ethylbenzene, and xylene). Other genetically engineered microbial biosensors have been developed for *in situ* detection of metals such

as cadmium, nickel, cobalt, different forms of mercury, arsenite, and other heavy metals such as copper, zinc, and lead (as summarized in Shin 2010).

Potentially, there could be a number of biosensor applications developed that would be relevant to agriculture that would be subject to review under TSCA. Future developments could include the use of intergeneric microorganisms as biosensors for detection of bioterrorist agents, detection of other environmental pollutants, including pesticides, some of which may have relevance to agriculture. Other potential agricultural uses could be development of microbial biosensors for detection of pathogenic strains of *E. coli* or *Salmonella* in the environment, for instance, in irrigation water, in soils, in manures and other fertilizers that are used for food crop production. These types of biosensors may be particularly useful for produce often consumed raw such as lettuces, spinach, onions, etc. However, a biosensor such as this, if used to monitor contamination on the actual food product rather than the environment in which the crop is growing, would fall under the jurisdiction of the FDA rather than EPA. Other agriculturally relevant future biosensors could be for monitoring nutrient or water status of soils or contamination of water used in crop production or in aquaculture.

Pesticidal Intermediates

Pesticidal intermediates are an agricultural application reviewed under TSCA, and several of these were reviewed in the 1980s. A pesticidal intermediate is a live microorganism producing a pesticide that contains only inactivated microorganisms. The final pesticide product containing dead microorganisms is reviewed by EPA's Office of Pesticide Programs under FIFRA. However, the live microorganism used in the production of the pesticide is reviewed under TSCA as a pesticidal intermediate. Future submissions of pesticidal intermediates may also be expected.

Weather Modification

Some of the earliest biotechnology applications involving intergeneric microorganisms involved those in weather modification. There was the ice-minus *Pseudomonas syringae* for prevention of frost damage on strawberries. The commercial product called Snomax is a strain of *P. syringae* that increases the nucleation temperature of water, thereby increasing snow volume. Since strains of *P. syringae* are known plant pathogens, USDA had the lead in reviewing these two products in the 1980s under the Plant Protection Act. However, any such weather modification product produced in the future using an intergeneric microorganism that did not fall under review by another federal agency would be reviewed under TSCA.

Algal Biomass for Fuels and Other Uses Such as Animal Feeds, Aquaculture Feed, Etc

Currently there are extensive R&D activities on using algae as a biofuel feedstock. Characteristics of microalgae production that are advantageous include high biomass

yields per acre, a lack of competition for arable land and sometimes nutrients, the use of waste water, produced water, or saline water, the recycling of carbon through use of CO_2 from industrial flue gas or other sources, and because production is compatible with an integrated biorefinery concept. Other aspects of microalgal culture include rapid growth rate, high cell density, and high oil content. Algae may be able to produce several fuel types including gaseous compounds like hydrogen and methane, as well as a range of conventional liquid hydrocarbons. Most of the current focus with algal biofuels is on the development of liquid transportation fuels including gasoline, diesel, and jet fuel.

The U.S. Department of Energy biofuels roadmap (US DOE 2010) addressed many aspects of this rapidly developing industry, including the variety of algal types, methods to cultivate them, and processes to recover oil from them. Algae can be grown photosynthetically using natural daylight or with artificial lighting. Heterotrophic algae can be grown much like other industrial microorganisms via continuous culture in the dark although when grown this way, they require a fixed carbon source such as sugars. There are two primary cultivation approaches with many variations. Photobioreactors utilize closed cycle recirculation systems employing either ambient light or artificial illumination. Open pond production facilities are generally raceway ponds of a recirculating design using pumps and paddle wheels to circulate water, algae, and nutrients through shallow open ponds. Hybrid systems growing algae in the environment may also be used, however perhaps with enclosures such as plastic bags, to contain the algae rather than growing them in the open.

Commercial fuel production from algae is in its infancy, but the growth of algae for commercial production of high-value end products such as pharmaceuticals and "nutraceuticals" has existed for some time. Products such as carotenoids, phycobilins, fatty acids, polysaccharides, vitamins, sterols, and biologically active molecules for use in human and animal health are produced by algae commercially (Oilgae 2010). Any intergeneric algae used for biofuel production would be reviewed under TSCA. Although these high-value end products other than fuels mentioned above would be reviewed by other federal agencies, TSCA would be involved if the algae were also producing biofuels.

4.7 Conclusions

Regulation of genetically engineered microbial agents, whether for pest management purposes or environmental bioremediation, has afforded the proper oversight of a novel technology as part of a larger attempt to reduce the uncertainty of the risk assessment process. With the advent of a new technology, uncertainties and lack of a proven track record necessitate thorough review of these microbes to ensure human health and environmental safety (Harrison and Bonning 2000). While the addition of a transgene to a familiar microbial genome may alter the phenotype of the microbe, these microbes are guided by the same biochemical and genetic processes as naturally occurring microbes (NRC 2000). Hence, they were assessed with that

fact in mind, albeit under an initial higher level of scrutiny and oversight. As indicated by Wrubel et al. (1997), decisions regarding further research and development of GE MPCA products may have been considerably influenced by unknowns in regulatory oversight, however, in the majority of cases an inability of the proposed product to live up to expectations was the driving force behind a products demise. One must not discount the perceived influence of public acceptance and its relationship to marketing of products, particularly when they involve food and feed.

Reports from the early field experiments with GE bacteria reveal how controversial and polarizing these first ventures were in the public arena (Griffin 1988; Berg and Singer 1995). Today this is largely not the case, although many have learned the value in public education and involvement in field testing novel technologies. It is still possible, however, to emote fear of the unknown without really intending to (Dixon 2008).

References

All JN, Stancil JD, Johnson TB, Gouger R (1994) A genetically-modified *Bacillus thuringiensis* product effective for control of the fall armyworm (Lepidoptera: Noctuidae) on corn. Fla Entomol 77:437–440

American Cyanamid (1994) Notification to conduct small-scale field testing a genetically altered baculovirus. EPA No. 241-NMP-2, EPA-OPP Public docket no. 50799

American Cynamid (1996) Notification to conduct small-scale field testing a genetically altered baculovirus. EPA No. 241-NMP-3, EPA-OPP Public docket no. 50816

Angle JS, Levin MA, Gagliardi JV, McIntosh MS (1995) Validation of microcosms for examining the survival of *Pseudomonas aureofaciens* (lacZY) in soil. Appl Environ Microbiol 61:2835–2839

Barinaga M (2000) Asilomar revisited: lessons for today? Science 287:1584–1585

Baum JA (1998) Transgenic *Bacillus thuringiensis*. Phytoprotection 79:127–130

Baum JA, Kakefuda M, Gawron-Burke C (1996) Engineering *Bacillus thuringiensis* bioinsecticides with an indigenous site-specific recombination system. Appl Environ Microbiol 62:4367–4373

Berg P, Singer MF (1995) The recombinant DNA controversy: twenty years later. Proc Natl Acad Sci 92:9011–9013

Bishop DHL (1988) Release of genetically altered viruses into the environment. Brit Med J 296: 1685–1686

BLR 741 (1988) Genetically engineered organisms – environmental release. Biotechnol Law Rep 7(1):20–96. doi:10.1089/blr.1988.7.20, Published in Volume: 7 Issue 1: March 20, 2009

Cory JS (2000) Assessing the risks of releasing genetically modified baculoviruses: progress to date. Crop Prot 19:779–785

Cory SJ, Hirst ML, Williams T, Hails RS, Goulson D, Green BM, Carty TM, Possee RD, Cayley PJ, Bishop DHL (1994) Field trial of a genetically engineered baculovirus insecticide. Nature 370:138–140

Crawford M (1986) Regulatory tangle snarls agricultural research the biotechnology arena. Science 234:275–277

de Doucet MMA, Miranda MB, Bertolotti MA (1998) Infectivity of entomogenous nematodes (Steinernematidae and Heterorhabditidae) to *Pediculus humanus capitis* De Geer (Anoplura: Pediculidae). Fund Appl Nematol 21:13–16

Dixon B (2008) Questionable experiments: the words used to describe some avenues of research may be so emotive outside of science as to foster acute anxiety in the wider world. Microbe, ASM News, pp 216–217, May 2008

DuPont (1996) Notification to conduct small-scale field testing a genetically altered baculovirus. EPA No. 352-NMP-4

Engler R (1974) Government regulation of microbial biopesticides. Dev in Indus Micro 15:199–207

EPA (1999) Status report: TSCA biotechnology submissions. Status report for fiscal years 87–97. http://www.epa.gov/oppt/biotech/pubs/pdf/bistat99.pdf. Accessed 24 June 2011

Federal Register (1994) Microbial pesticides; experimental use permits and notifications; final rule, environmental protection agency, 59, No. 169, 45612, Thursday, 1 Sept 1994. http://www.gpo.gov/fdsys/pkg/FR-1994-09-01/html/94-21358.htm. Accessed 24 May 2011

Federal Register (1995) CryIA(c) and CryIC derived delta endotoxins of *Bacillus thuringiensis* encapsulated in killed Pseudomonas fluorescens; exemption from the requirement of a tolerance, vol 60. Environmental Protection Agency, FR, No. 177, 47487–47489, Wednesday, 13 Sept 1995. http://www.gpo.gov/fdsys/pkg/FR-1995-09-13/pdf/95-22617.pdf. Accessed 28 July 2011

Federal Register (1997) Receipt of notifications to conduct small-scale field testing of genetically engineered microbial pesticides, Environmental Protection Agency, FR 62(83):23448–23449, Wednesday, 20 April 1997. http://www.gpo.gov/fdsys/pkg/FR-1997-04-30/html/97-11021.htm. Accessed 21 Dec 2011

Federal Register (2007) 40 CFR Part 158 Pesticides; data requirements for biochemical and microbial pesticides FR 72(207):60988–61025, Friday, 26 Oct 2007. http://edocket.access.gpo.gov/2007/pdf/E7-20828.pdf. Accessed 1 Oct 2010

Folimonov AS, Folimonova SY, Bar-Joseph M, Dawson WO (2007) A stable RNA virus-based vector for citrus trees. Virology 368:205–216

Gagliardi JV, Angle JS, Germida JJ, Wyndham RC, Chanway CP, Watson RJ, Greer CW, McIntyre T, Yu HH, Levin MA, Russek-Cohen E, Rosolen S, Nairn J, Seib A, Martin-Heller T, Wisse G (2001) Intact soil-core microcosms compared with multi-site field releases for pre-release testing of microbes in diverse soils and climates. Can J Microbiol 47:237–252

Gard IE, Treacy MF, Wrubel JJ (2002) Case study: recombinant baculoviruses as microbial pesticidal agents. In: Atherton KT (ed) Genetically modified crops: assessing safety, Taylor and Francis, pp 201–218

Gaugler R, Wilson M, Shearer P (1997) Field release and environmental fate of a transgenic entomopathogenic nematode. Biol Control 9:75–80

Gillespie KM, Angle JS, Hill RL (1995) Runoff losses of *Pseudomonas aureofaciens* (lacZY) from soil. FEMS Microbiol Ecol 17(4):239–245

GMO Compass (2010) Overview: foods, genetic engineering, and labeling. http://www.gmocompass.org/eng/grocery_shopping/processed_foods/26.overview_foods_genetic_engineering_labelling.html. Accessed 29 Dec 2010

Grewal PS, Peters A (2005) Formulation and quality. In: Grewal PS, Ehlers RU, Shapiro DI (eds) Nematodes as biocontrol agents. CABI Publishing, Wallingford, pp 79–90

Griffin K (1988) Public fears factored into gene-altered bacteria tests. Los Angeles Times, 18 April 1988. http://articles.latimes.com/1988-04-18/local/me-992_1_altered-bacteria. Accessed 29 Dec 2011

Harrison RL, Bonning BC (2000) Genetic engineering of biocontrol agents for insects. pp 305–357. In: Rechcigl JE, Rechcigl NA (eds) Biological and biotechnological control of insect pests, pp 243–280, CRC Press, ISBN 1-56670-479-0

Hashmi S, Hashmi G, Gaugler R (1995) Genetic transformation of an entomopathogenic nematode by microinjection. J Invertebr Pathol 66:293–296

Head J, Walters KFA, Langton S (2000) The compatibility of the entomopathogenic nematode, *Steinernema feltiae*, and chemical insecticides for the control of the South American leafminer, *Liriomyza huidobrensis*. BioControl 45:345–353

Heinz K, McCutcheon BF, Herrmann R, Parrella MP, Hammock BD (1995) Direct effects of recombinant nuclear polyhedrosis viruses on selected nontarget organisms. J Econ Entomol 88:259–264

Hoover K, Herrmann R, Moskowitz H, Bonning B, Duffey SS, McCutchen BF, Hammock BD (1996) Bioinsecticides: the potential of recombinant baculoviruses as enhanced bioinsecticides. Pestic Outlook 7:21–27

Hu G, St. Leger RJ (2002) Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. Appl Env Microbiol 68:6383–6387

Hughes PR, Wood HA, Breen JP, Simpson SF, Duggan AJ, Dybas JA (1997) Enhanced bioactivity of recombinant baculoviruses expressing insect-specific spider toxins in Lepidopteran crop pests. J Invert Pathol 69:112–118

Inceoglu AB, Kamita SG, Hammock BD (2006) Genetically modified baculoviruses: a historical overview and future outlook. Adv Virus Res 68:323–360

Jones ME (1999) Politically corrected science, the early negotiation of U.S. agricultural biotechnology policy. Ph.D. dissertation submitted to Virginia Polytechnic Institute and State University. http://scholar.lib.vt.edu/theses/available/etd-120199-091346/unrestricted/MEJ-etd-modified.pdf. Accessed 24 May 2011

Kleupfel DA, Kline EL, Skipper HD, Hughes TA, Gooden DT, Drahos DJ, Barry GF, Hemming BC, Brandt EJ (1991) The Release and tracking of genetically engineered bacteria in the environment. symposium: assessing the socioeconomic, ecological, and scientific effects of agricultural biotechnology. Phytopathology 81:348–352

Kunimi Y, Fuxa JR, Hammock BD (1996) Comparison of wild type and genetically engineered nuclear polyhedrosis viruses of Autographa californica for mortality, virus replication and polyhedral production in Trichoplusia ni. Entomol Exp Appl 81:251–257

Lampel JS, Canter GL, Dimock MB, Kelly JL, Anderson JJ, Uratani BB, Foulke JS, Turner JT (1994) Integrative cloning, expression, and stability of the cryl4(c) gene from *Bacillus thuringiensis* subsp. *kurstaki* in a recombinant strain of *Clavibacter xyli* subsp. Cynodontis. Appl Env Microbiol 60:501–508

Lindow SE (1985) Ecology of *Pseudomonas syringae* relevant to use of Ice – deletion mutants constructed in vitro for plant frost control. In: Halvorson HO, Pramer D, Rogul M (eds) Engineered organisms in the environment: scientific issues. American Society for Microbiology, Washington, DC, pp 23–25

Lindow SE, Panopoulos NJ (1988) Field tests for recombinant Ice-*Pseudomonas syringae* for biological frost control in potato. In: Sussman M, Collins CH, Skinner FA, Stewart-Tull DE (eds) The release of genetically engineered microorganisms. Academic, New York, pp 121–138

Marchant GE (1988) Modified rules for modified bugs: balancing safety and efficiency in the regulation of deliberate release of genetically engineered microorganisms. Harvard J Law Technol 1:163–208

Martin WR (1997) Using entomopathogenic nematodes to control insects during stand establishment. Hort Sci 32(2):196–200

Marvier M, McCreedy C, Regetz J, Kareiva P (2007) A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. Science 316:1475–1477

Maugh TH, II (1987) Altered bacterium does its job: frost failed to damage sprayed test crop, company says. LA Times 09 June 1987. http://articles.latimes.com/1987-06-09/news/mn-6024_1_frost-damage. Accessed 21 Dec 2011

McLintock JT, van Beek, NAM, Kough, JL, Mendelsohn, ML, Hutton, PO (2000) Regulatory aspects of biological control agents and products derived from biotechnology. In: Rechcigl JE, Rechcigl NA (eds) Biological and biotechnological control of insect pests, CRC Press, Boca Raton, pp 305–357, ISBN 1-56670-479-0

Milewski E (1987) The NIH guidelines and field testing of genetically engineered plants and microorganisms in application of biotechnology: environmental and policy issues. In: Fowle JR, III (ed.) Westview Press, for the American Association for the Advancement of Science, Boulder, CO. pp 55–90. See p 63

Mycogen (1998) Mycogen corporation: company perspectives. http://www.fundinguniverse.com/company-histories/Mycogen-Corporation-company-History.html. Accessed 21 Nov 2011

Navon A (2000) *Bacillus thuringiensis* insecticides in crop protection – reality and prospects. Crop Prot 19:669–676

New Scientist (1986) Engineered organisms tested on the roof. New Scientist, p 24, 6 Mar 1986
NIH (1976) NIH recombinant DNA research guidelines July 7, 1976. Fed Reg 41:27902
NIH (2011) NIH guidelines for research involving recombinant DNA molecules. http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.pdf . Accessed 11 June 2011
NRC (National Research Council) (2000) Executive Summary, pp 5–6, In: Genetically Modified Pest Protected Plants: Science and Regulation, National. Academy Press, Washington, D.C., USA, ISBN 0-309-06930-0
O'Reilly DR, Miller LK (1989) A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. Science 245:1110–1112
O'Reilly DR, Miller LK (1991) Improvement of a baculovirus pesticide by deletion of the *egt* gene. Biotechnology 9:1086–1089
Obukowicz MG, Perlak FJ, Kusano-Kretzmer K, Mayer EJ, Watrud LS (1986) Integration of the delta-endotoxin gene of *Bacillus thuringiensis* into the chromosome of root-colonizing strains of pseudomonads using Tn5. Gene 45(3):327–331
Oilgae Newsletter (15 Sept 2010). http://www.oilgae.com/blog/2010/09/algae-as-a-source-of-pharmaceuticals-nutraceuticals.html. Accessed 24 June 2011
OSTP (1986) Coordinated framework for regulation of biotechnology. 51 FR 23302–93, 26 June 1986. http://usbiotechreg.nbii.gov/. Accessed 22 Nov 2010
OSTP (2001) SIDEBAR No. II.A, BIOCONTROL USING A VIRUS (AcMNPV) – following the Bt-maize case study. http://www.whitehouse.gov/files/documents/ostp/Issues/ceq_ostp_study3.pdf. Accessed 22 Nov 2010
OTA, Office of Technology Assessment (1988) New developments in biotechnology-field-testing engineered organisms: genetic and ecological issues, p 51, OTA-BA-350, U.S. Government Printing Office, Washington, DC, May 1988. http://www.fas.org/ota/reports/8816.pdf. Accessed 18 Oct 2011
OTA, Office of Technology Assessment (1995) Biologically based technologies for pest control, U.S. Congress, Office of Technology Assessment, OTA-ENV-636, U.S. Government Printing Office, Washington, DC, September 1995. http://www.fas.org/ota/reports/9506.pdf. Accessed 24 Oct 2011
Pizzuli E (1984) Foundation on economic trends v. Heckler: genetic engineering and NEPA's EIS requirement. Pace Env Law Review 2:138–165. http://digitalcommons.pace.edu/pelr/vol2/iss1/7 accessed 070111
Rizvi SA, Hennessey R, Knott D (1996) Legislation on the introduction of exotic nematodes in the US. Biocontrol Sci Technol 6:477–480
Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnol J 9:283–300
Schneider W (2010) Personal communication, U.S. Environmental Protection. Agency, Office of Pesticide Programs (retired), 21 October 2010
Selçuk H, Kaya HK, Stock SP, Keskün N (2003) Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of soil pests. Turk J Biol 27:181–202
Shand H (1989) Bacillus thuringiensis : industry frenzy and a host of issues. JPesticide Reform 9:18–21. http://eap.mcgill.ca/MagRack/JPR/JPR_05.htm. Accessed 19 Oct 2011
Shin HJ (2010) Genetically engineered microbial biosensors for in situ monitoring of environmental pollution. Appl Microbiol Biotechnol, On-line publication DOI 10.1007/s00253-010-2990-8, Springer-Verlag, 10 Nov 2010
Slavicek JM, Popham H, Riegel CI (1999) Deletion of the *Lymantria dispar* multicapsid nucleopolyhedrovirus ecdysteroid UDP-glucosyl transferase gene enhances viral killing speed in the last instar of the gypsy moth. Biol Control 16:91–103
Smith FL, Jr (1997) Prometheus bound: cloning bears identical reactions. Regulation 20(2). http://www.cato.org/pubs/regulation/regv20n2/reg20n2j.html. Accessed 21 Nov 2011
St. Leger RJ, Joshi L, Bidochka MJ, Roberts D (1996) Construction of an improved mycoinsecticide overexpressing a toxic protease. Proc Natl Acad Sci 93:6349–6354
Summers MD (2006) Milestones leading to the genetic engineering of baculoviruses as expression vector systems and viral pesticides. Adv Virus Res vol 68 Elsevier DOI: 10.1016/S0065-3527(06)68001-9. http://www.tamu.edu/faculty/summers/publications/milestones.pdf . Accessed 18 Oct 2011

Supkoff DM, Bezark LG, Opgenorth D (1988) Monitoring of the 1987 winter field release of genetically engineered bacteria in Contra Costa county. Biological Control Services Program, Department of Food and Agriculture, State of California. http://www.cdpr.ca.gov/docs/pest-mgt/pubs/bacteria_contra_costa.pdf. Accessed 21 Dec 2011

Tang X, Sun X-L, Pu G-Q, Wang W-B, Zhang C-X, Zhu J (2011) Expression of a neurotoxin gene improves the insecticidal activity of *Spodoptera litura* nucleopolyhedrovirus (SpltNPV). Virus Res 59:51–56

Tomalski MD, Miller LK (1991) Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. Nature 352:82–85

Tomalski MD, Kutney R, Bruce WA, Brown MR, Blum MS, Travis J (1989) Purification and characterization of insect toxins derived from the mite, *Pyemotes tritici*. Toxicon 27:1151–1167

Turner JT, Lampel JS, Stearman RS, Sundin GW, Gunyuzlu P, Anderson JJ (1991) Stability of the δ-endotoxin gene from *Bacillus thuringiensis* subsp. kurstaki in a recombinant strain of *Clavibacter xyli* subsp. *Cynodontis*. Appl Environ Microbiol 57:3522–3528

Turner JT (2011) Personal communication. Biotechnology Regulatory Services. Animal and Plant Health Inspection Service, U.S. Department of Agriculture, 15 November 2011

U.S. DOE (2010). National algal biofuels technology roadmap. U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Biomass Program. Washington, DC. May, 2010

Van J (1989) In oil-spill cleanups, major tool off limits. Chicago Tribune. http://articles.chicagotribune.com/1989-06-18/news/8902100449_1_microbes-oil-spill-cleanups-oil-spill. Accessed 09 Nov 2011

Wang C, St. Leger RJ (2007) A scorpion neurotoxin increases the potency of a fungal insecticide. Nat Biotech 25:1455–1456

Watrud LS, Perlak FJ, Tran MT, Kusano K, Miller-Wideman MA, Obukowicz MG, Nelson DR, Kreitinger RJ, Kaufman JP (1985) Cloning of the *Bacillus thuringiensis* subsp. *kurstaki* delta-endotoxin gene into *Pseudomonas fluorescens*: molecular biology and ecology of an engineered microbial pesticide. In: Halvorson HO, Pramer D, Rogul M (eds) Engineered organisms in the environment: scientific issues. American Society for Microbiology, Washington, DC, pp 40–46

Weinzierl R, Henn T, Koehler PG, Tucker CL (2005) Microbial insecticides. Entomology and Nematology Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. http://edis.ifas.ufl.edu/pdffiles/IN/IN08100.pdf . Accessed 25 Oct 2011

Wilson M, Xin W, Hashmi S, Gaugler R (1999) Risk assessment and fitness of a transgenic entomopathogenic nematode. Biol Control 15:81–87

Wrubel RP, Krimsky S, Anderson MD (1997) Regulatory oversight of genetically engineered microorganisms: has regulation inhibited innovation? Environ Manage 21:571–586

Chapter 5
The Status, Promise and Potential Perils of Commercially Available Genetically Modified Microorganisms in Agriculture and the Environment

Anne K. Vidaver, Sue Tolin, and Angela Post

Abstract Modern genetics has shown the power of modifying microbes, from viruses to bacteria to algae, to produce desirable agricultural products. Nevertheless, gene additions or modification have led to relatively few products in the marketplace due partly to costs of regulation, but also to the challenges of production, delivery and application. Some products with gene loss have been marketed, notably *Agrobacterium radiobacter* with a deletion for plasmid transfer, some veterinary vaccines and plants with one or a few genes from microbes for plant protection. Concerns over using live microbes are centered on recombination with wild type strains, potential for environmental risks, market acceptance, market scope, monitoring costs, and costs of production. The challenges in microbial agricultural plant biotechnology far outweigh those in medical and veterinary biotechnology because of pricing potential, larger markets and controlled environments in which modified microbes can function. Nevertheless, the promise and need for control of plant pathogens for which little or no plant resistance is available warrant continued efforts in this area. Veterinary uses of modified microbes will continue and be more widely accepted. Plants 'vaccinated' with genes for plant protection are increasingly used but their safety is still questioned and debated. Products such as enzymes from GMOs will continue to enter the marketplace and be accepted with few questions.

Keywords Genetic engineering • Commercial products • PIPs • Biocontrol • Vaccines

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5.1 Introduction

Genetically modified microorganisms (GMOs) based on recombinant DNA techniques have been constructed since the 1970s. The potential for beneficial use was recognized very early, as was their potential risk through misuse, intentional or accidental. This dilemma remains with us in the twenty-first century. GMOs have also been known as genetically engineered microorganisms (GEOs) or genetically modified microbes (GMMs). Many of the issues related to the use of engineered microbes in agriculture have been presented earlier (Ryder 1994; Wilson and Lindow 1993; Wrubel et al. 1997), in a symposium on '*The Scientist's Role in the Controversy Over Genetic Engineering, Regulation and Utilization of Microorganisms*' (Vidaver 1989), and more recently in a study by the National Academy of Sciences (NRC 2004). However, the bulk of these presentations and discussions appeared two decades or more ago in the early days of modern genetics and genetic engineering. Today, the ease and lower cost of nucleic acid sequencing for genome analysis, improved methods of detection of microorganisms and specific sequences, new discoveries in genetic manipulation, and synthetic biology raise new issues to ponder and new approaches to assessing microbial ecology and the risks and benefits of GMOs in agriculture. In this chapter, we deal principally with commercialization of products used for plant and animal production and protection in agriculture.

5.2 Current Status of GMOs

5.2.1 Regulation

GMOs are regulated in the U.S. based on the intended use, whereas in Canada regulation is a part of novel product oversight. Microbial GMOs in plant agriculture agents, ranging from viroids to nematodes (Table 5.1), are under the jurisdiction of the Environmental Protection Agency (EPA) (see elsewhere in this book) if the objective of their use is for pest control. In Canada jurisdiction is by Health Canada. Protective veterinary products for animals and fish are regulated and licensed by the

Table 5.1 Relative potential of microorganisms as GMOs for use in agriculture

Agent	Ease of genetic manipulation	Ease of production	Ease of application
Viroids	Variable	Challenging	Difficult
Viruses	Variable	Challenging	Challenging
Bacteria	Variable	Easy	Easy
Fungi	Challenging	Challenging	Challenging
Oomycetes	Difficult	Difficult	Difficult
Protozoa	Difficult	Difficult	Difficult
Algae	Difficult	Challenging	Difficult
Nematodes	Difficult	Difficult	Variable

Center for Veterinary Biologics of the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) in the U.S. and by the Canadian Food Inspection Agency (CFIA) in Canada. APHIS also has authority under the Plant Protection and Quarantine program over introduction and release into the environment of organisms that are or may be plant pests.

5.2.2 Commercial Product Overview

GMOs have a minor use in agriculture. Products are limited because of costs of regulation and marketplace determination of availability and applications. Since 1976, the U. S. EPA has compiled an inventory of about 82,000 chemicals produced in or imported into the U.S., many of which are used in agriculture (Schierow 2009). That period of over-three decades coincides with the rise of modern genetics and tools for modification of microorganisms. To date, there are 63 commercial products that we have identified, comprising living microbes, plants modified with microbial genes for pest protection, and veterinary vaccines. There are also a couple dozen enzymes derived from GMOs used in the food industry. This is hardly a robust number and cause for concern or alarm.

Bacteria, fungi and viruses are the major candidates of choice for genetic manipulation. Commercial products for protection could be in any form, but the most common is with the use of live or non-viable agents applied for competitive exclusion or direct competition for receptor sites on plant parts (Wilson and Lindow 1994), or as vaccines in animals (Jackwood et al. 2008; Meeusen et al. 2007). All of the agents approved thus far are primarily effective when used prior to exposure of the infectious agent. About 22 enzymes derived from GMOs are used in the food industry worldwide (Olempska-Beer et al. 2006).

5.2.3 Vaccines in Animal Agriculture

There are a number of genetically engineered veterinary viral and bacterial vaccines, including gene-deleted vaccines and live recombinant chimera viruses that combine parts of two infective viral genomes (Jackwood et al. 2008; Meeusen et al. 2007). These vaccines are in three categories: live genetically modified microbes (viruses or bacteria with one or more genes deleted or inactivated or carrying a foreign gene), recombinant inactivated vaccines (subunit vaccines containing only part of the whole organism) and genetic vaccines (nucleic acids or DNA with foreign genes). There are 21 of these commercially available for treatment of a wide variety of animals: ruminants, swine, poultry and companion animals. However, these are still a minor part of the commercial vaccine market. As of 2010, the USDA Center for Veterinary Biologics has listed 28 vaccines categorized as seven non-replicating recombinant antigen vaccines, two nucleic acid-mediated vaccines, four live gene-deleted vaccines and 15 live vectored vaccines.

Table 5.2 Registered genetically modified microbes and primary use in plant agriculture

Microorganism	Use
Agrobacterium radiobacter K1026	Protection of roots from crown gall
Pseudomonas fluorescens (killed) with *Bacillus thuringiensis* delta endotoxins (endotoxins from B.t. strains aizawai, Kurstaki or San Diego)	Insecticidal spray (no reproduction of host bacterium)

5.2.4 Microorganisms Associated with Plants and Plant Pests

A small number of free-living GMOs have been approved for use since the last century (Amarger 2002); only those in current use are listed in Table 5.2. These have a Biopesticide Regulatory Action Document (BRAD) that indicates current status in the U.S. Note that strains are specifically mentioned. For example *Agrobacterium radiobacter* K1026 (Jones and Kerr 1989) is a Tra- (transfer negative) derivative of *A. radiobacter* K84, a naturally occurring bacterium effective against crown gall, caused by a tumorigenic relative, *A. tumefaciens*. A transferable plasmid in K84 also carries a gene for a specialized antibiotic or bacteriocin effective against *A. tumefaciens*. Deletion of the Tra+gene prevents the rare transfer to *A. tumefaciens*, which could make it resistant to biocontrol. We were unable to find records of GMOs used outside the U.S., except for Australia where *A. radiobacter* K1026 originated; it has been used commercially since 1988 for reducing crown gall infection of stone fruits, such as peach and cherry and ornamentals, notably roses (Ryder 1994).

In the case of the *Bacillus thuringiensis* (Bt) derivatives, each has differing insecticidal properties. The corresponding wild-type (natural) strains have been used for about 70 years, including in organic farming. The modified strains or host bacteria attach to plant receptors more easily and are more resistant to UV light degradation than the parent strains. The host bacterium, dead or alive, has no known deleterious effects on animals, plants or humans. Extensive information on the analysis conducted by EPA of *Bt* in several formulations has been summarized by Mendelsohn et al. (2003).

There are a miniscule number of potential products that could provide protection for plants from infectious agents. The historic experiments with a *Pseudomonas syringae* ice-minus deletion summarized by Lindow (1989) and Wilson and Lindow (1993) did not lead to a viable product, although an unmodified strain is used now to protect plants from frost under a narrow temperature range. There was a transient commercialization of *Sinorhizobium* (*Rhizobium*) *meliloti* RMSPC-2 (EPA 1997, 1998) as seed inoculants for alfalfa. The strain had genes to enhance nitrogen fixation and nutrient utilization, as well as an antibiotic resistant marker gene (http://epa.gov/biotech_rule/pubs/factdft6.htm). The commercial transfer of the Bt delta endotoxin gene to the endophyte *Clavibacter xyli* for control of the corn ear worm (Tomasino et al. 1995) lost to competition with the development of Bt genes transformed as integral parts of the plant cell.

5.2.5 *Microbial Genes as Plant Protectants*

Microbial genes inserted into plants have been widely adopted since the mid-1990s. In the U.S., these genes for plant protection are classified as 'PIPs' or plant-incorporated protectants, of which there are now about 40 registered with the EPA (http://www.epa.gov/oppbppd1/biopesticides/index.htm#pips). The majority use genes from Bt strains for insect control (Mendelsohn et al. 2003). Virus-protected papaya and cucurbit plants have been commercialized for several years but are not listed on the PIP website. The latest candidate is the coat protein gene of *Plum pox virus* used to protect stone fruit trees which has recently been approved for commercialization (See Chap. 12) (http://www.epa.gov/oppbppd1/biopesticides/ingredients/tech_docs/brad_006354.pdf).

5.3 Constraints

Not surprisingly, the use of dead or inactivated microbes (e.g. vaccines for animals) has been more widely accepted and commercialized than the use of live microbes or chemicals for plant protection (Table 5.3). Microbes are likely to be viewed more negatively in agriculture if they are able to replicate. Questions continue to be raised about their survival, persistence, contamination, spread, efficacy of expression of the beneficial trait(s), and gene transfer. This is the case even though no substantive differences have been found between GMOs and the corresponding parent strains (Amarger 2002; Wilson and Lindow 1994). Although substantial equivalence is becoming accepted for food safety/risk assessments (LeBlanc et al. 2010), we believe it is less likely to be used for agricultural and environmental applications.

5.4 Promises and Perils

Many opportunities and challenges remain. Containment remains an issue, but bioconfinement of microorganisms is possible genetically and physically (NRC 2004). Microbiologists and ecologists with little experience with plant associated microbes

Table 5.3 Strategies and attributes of introduced microorganisms and chemicals used in plant health and protection

Strategy/attribute	Microorganisms	Chemicals
Replication	Yes (limited)	No
Shelf-life	Variable	Long
Ecological contamination	Rare	Variable
Cost (research, production, regulatory)	Variable	High
Persistence	Rare	Variable
Specificity	Common	Rare
Safety	Absolute (?); no reported adverse effects	Variable
Market prospects	Relatively limited	Wide

remain concerned about reproduction, survival, and gene transfer to and from other microbes. When these questions have been dealt with experimentally and data provided for risk assessment, many candidate microbes may or may not be considered suitable for use. It is seldom recognized that thousands to millions of microbes colonize plants, including imported inspected plants and bulbs, and most do so in a beneficial or neutral manner. Even so, the public is reluctant to use microorganisms, compared with chemicals, because of the greater familiarity with germ and human disease causality rather than with the beneficial role of microbes in the environment (Table 5.3).

For animals, bio-engineered vaccines show great promise through using reverse genetics, non-replicating viral vectors, cytoplasmic replicating viruses (alpha viruses; positive stranded RNA viruses) and genetic vaccines, as well as benefiting from improved adjuvants and delivery systems (Patel and Heldens 2009).

New challenges and opportunities also lie with synthetic biology. For example, viruses can be readily constructed *de novo* from commercially available nucleotides, and a partially synthetic bacterium has been constructed. Due to the high monetary costs of research and regulation, such constructs are not likely to be available in the agricultural sector in the near future. However, there is promise through plant genomics and limiting pathogen invasion through novel resistance genes and RNAi approaches.

5.4.1 Challenges

Taxonomy is also a challenge to microbial production and use, and in risk assessment. The scope of GMO regulation targets ‘intergenerics’, even though not all members of the same genus have similar habitats and traits. The use of taxa that include human and /or animal pathogens (e.g. *Burkholderia cepacia*) has met with opposition by several groups, even when there is no evidence of the strain’s ability to cause harm. And, whether *Rhizobium* (*Bradyrhizobium*) inoculants that receive transgenes from other members of the species should be regulated under the Toxic Substances Control Act remains an open question, as it defines GMOs as intergeneric. This example is particularly pertinent because of its close taxonomic relationship to *Agrobacterium*, a genus composed largely of plant pathogens.

A number of critical needs must be met before more products are available and used in agriculture, including potential production of biofuels using GMOs (Glass 2008). It would be helpful to categorize microbes according to risk groups and show that there are many that are generally regarded as compatible with the environment. A GRACE classification (Generally Regarded as Compatible with the Environment) would demonstrate to the public that the commercial strains are in such a group, such as *Rhizobium* and *Bradyrhizobium*, among others. There should be clear differentiation between fears and risks. Risk assessments should be based on available science and, naturally, regulations and guidelines should be commensurate with the risk. For small markets or specialty products, the equivalent of ORPHAN status might be considered. Delivery methodology needs to be improved in the plant

sector, as is being done with human and animal medical vaccines. New technologies such as synthetic biology and nanotechnology need to be evaluated for safe introduction into the environment. And public and media education is essential.

Clearly, the marketplace for the private sector has been uneven. Few prosper with live GMOs. This appears to be largely due to insufficient sales commensurate with perceived usefulness by the applicator and regulatory costs and constraints. The likelihood of increased numbers of free-living products in plant agriculture, based on 35 years of product analysis, is not promising. Public acceptance of transgenes in the products themselves has been widespread, but continues to be challenged by certain sectors including organic foods. More research and education in multiple forums may alleviate such fears and enable more product development.

References

Amarger N (2002) Genetically modified bacteria in agriculture. Biochimie 84:1061–1072

EPA (1997) Fact sheet: commercialization of *Sinorhizobium* (*Rhizobium*) *meliloti*, RMBPC-2. http://www.epa.gov/biotech_rule/pubs/factdft6.htm. Accessed 3 June 2011

EPA (1998) Final rule: *Sinorhizobium meliloti* strain RMBPC-2: significant new use rule. Fed Regist 63:29646–29648

Glass DJ (2008) Impact of government biotechnology regulations on biofuel development. CleanTech 2008, CSI Events, Boston

Jackwood MW, Hickle L, Kapil S, Silva R (2008) Vaccine development using recombinant DNA technology. Animal Agriculture's Future through Biotechnology, Part 7. CAST Issue paper 38, 12 pp. (www.cast-science.org)

Jones DA, Kerr A (1989) Agrobacterium radiobacter strain K1026, a genetically engineered derivative strain of strain K84, for biological control of crown gall. Plant Disease 73:15–18

LeBlanc JG, van Sinderen D, Hugenholtz J, Piard J-C, Sesma F, de Giori GS (2010) Risk assessment of genetically modified lactic acid bacteria using the concept of substantial equivalence. Curr Microbiol 61:590–595

Lindow SE (1989) Release and behavior of recombinant bacteria in field studies. J Iowa Acad Sci 96:71–73

Meeusen ENT, Walker J, Peters A, Pastoret P, Jungersen G (2007) Current status of veterinary vaccines. Clin Microbiol Rev 20:489–510

Mendelsohn M, Kough J, Vaituzis Z, Matthews K (2003) Are *Bt* crops safe? Nat Biotechnol 21: 1003–1009

National Research Council (U.S.). Committee on Biological Confinement of Genetically Engineered Organisms (2004) Biological confinement of genetically engineered organisms. National Academies Press, Washington, DC, 255 pp. (www.nap.edu)

Olempska-Beer ZS, Merker RI, Ditto MD, DiNovi MJ (2006) Food-processing enzymes from recombinant microorganisms- a review. Regul Toxicol Pharmacol 45:144–158

Patel JR, Heldens JGM (2009) Immunoprophylaxis against important virus diseases of horses, farm animals and birds. Vaccine 27:1797–1810

Ryder M (1994) Key issues in the deliberate release of genetically-manipulated bacteria. FEMS Microbiol Ecol 15:139–145

Schierow L-J (2009) The Toxic Substances Control Act (TSCA): implementation and new challenges. Congressional Research Service, 7–5700, 35 pp

Tomasino SF, Liester RT, Dimock MB, Breach RM, Kelly JL (1995) Field performance of *Clavibacter xyli* subsp. *cynodontis* expressing the insecticidal protein gene crylA (c) of *Bacillus thuringiensis* against European corn borer in field corn. Biological Control 5:442–448

Vidaver AK (1989) Public policy on the introduction of genetically engineered microorganisms. J Iowa Acad Sci 96:74–77
Wilson M, Lindow SE (1993) Release of recombinant microorganisms. Annu Rev Microbiol 47: 913–944
Wilson M, Lindow SE (1994) Ecological similarity and coexistence of epiphytic ice-nucleating (Ice^+) *Pseudomonas syringae* strains and a non-ice-nucleating (Ice^-) biological control agent. Appl Environ Microbiol 60:3128–3137
Wrubel RP, Krimsky S, Anderson MD (1997) Regulatory oversight of genetically engineered microorganisms: has regulation inhibited innovation? Environ Manage 21:571–586

Chapter 6
Regulatory Experiences in Symbiotic Control of Pierce's Disease

Thomas A. Miller

Abstract The amount of research funds for addressing practical plant pest or disease problems is determined by the value of the industry and the size of the threat. When new products, tools or strategies are developed, regulatory agencies must judge and evaluate these new tools that are not specifically described in the statutes that guide their decisions. Regulatory costs to pay for risk assessments make commercial sense when projected income from a new product can be charged against an investment based on the size of the expected market. When a pest or disease problem affects a minor crop, the research to address regulatory issues does not have such a clear-cut funding origin. Ironically, a very selective biopesticide designed to address a local pest or disease problem is the ideal form of sustainable pest management, but has the smallest market of any pest control strategy and therefore the smallest amount of financial support. In this sphere of modest financial resources, regulatory needs can force research away from solving the problem at hand to address unfocused or ill-defined risk issues. When genetic modification is a part of the proposed new strategy, an added burden is placed on the developers. This burden can defy logic and can, ironically, come largely from peers, not the public.

Keywords Competitive displacement • *Homalodisca vitripennis* • Pierce's disease • Symbiotic control • *Xylella fastidiosa* • *Alcaligenes xylosoxidans* var. *denitrificans*

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Acronyms

APHIS	Animal Plant Health Inspection Service
Axd	*Alcaligenes xylosoxidans* var. *denitiricans*
BPPD	Biopesticides and Pollution Prevention Division (of EPA)
BRS	Biotechnology Regulatory Service
BSC	BioSafety Committee (mandated at institutions receiving federal funds)
CDFA	California Department of Food and Agriculture
CF	Cystic Fibrosis
CFR	Code of Federal Regulations (http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&tpl=%2Findex.tpl)
DsRed	Red fluorescent dye widely used as a marker gene in molecular biology
ELISA	Enzyme-Linked ImmunoSorbant Assay
EPA	Environmental Protection Agency (of USA)
GWSS	Glassy-Winged Sharpshooter (*Homalodisca vitripennis;* originally *H. coagulata*)
OPP	Office of Pesticide Programs (of EPA)
PB	Bacteria of the genus *Paenibacillus* reported to protect plants against drought
PD	Pierce's Disease (of grapevines)
PPQ	Plant Pest Quarantine (a division of APHIS)
RAxd	Axd with DsRed marker gene inserted
SAP	Scientific Advisory Panel
TERA	TSCA Experimental Release Application
TSCA	Toxic Substances Control Act (administered by EPA)
USDA	United States Department of Agriculture
VS	Veterinary Services
Xf	*Xylella fastidiosa*

6.1 Introduction

A new pest or disease arrives in California every 60 days. The usual response to each new threat is the same, assessment, treatment and management. The initial threat is first: identified; a treatment with existing products is determined and while the initial threat is held at bay with tools that can be used immediately, a longer term solution is sought. The size of the economic threat to the industry affected determines the funding available to mount counter-measures. The response to all such threats fades over time as new threats demand attention.

In some cases longer term solutions are never found and the interim treatment phase becomes semi-permanent with initial emergency funding from state government replaced by longer-term financing from the affected commodity group. Three of the longest-running programs of this kind in California are control of beet leafhooper, *Circulifer tenellus*, to prevent Curly Top Virus of vegetables, roguing (removal) of diseased citrus trees to prevent establishment and spread of Tristeza virus, and sterile

insect control of pink bollworm. Boards and Agencies were established for each of these programs that provided the legal framework to raise funds from commodity sales to pay yearly costs and provide legal rights to remove diseased trees for the greater good. The Curly Top Virus Control Board; Central California Tristeza Virus Eradication Agency; and California Cotton Pest Control Board respectively are examples of these legal organizations.

The response paradigm of assess, treat and manage does not reveal how few options are available to respond to a new pest invasion. For example as many as 100,000 acres of range and idle agricultural lands in California are treated with insecticides to control beet leafhopper before spring populations carry virus to susceptible crops in the Central Valley of California. Despite hundreds of thousands of dollars spent on biological control, no effective parasites have been located to help with population suppression. Thus the program remains in the extended treatment phase (http://www.cdfa.ca.gov/phpps/ipc/curlytopvirus/ctv_hp.htm).

The same principal of assess, treat and manage, described above, also operated in the recent swine flu mini-epidemic that first appeared in Mexico (with the actual origin still unknown) and saturated the media in Winter-Spring of 2009. The first response was to identify the virus (Enserink 2009). Next, monitoring and quarantine methods were put in place for detection and to isolate affected individuals to stop the spread (just like Tristeza virus in citrus). Eventually the severity of the reported cases declined and interest faded. It is interesting in the aftermath of the Swine flu incident that scientists wound up speculating about the rate and virulence of mutations. This speculation occurs due to a lack of understanding of how the viruses or pathogens are created and lack of tools to deal with them.

Indeed, at issue in all new biological threats such as these is the paucity of tools. Since new methods of gene manipulation provide an entirely new way of developing new tools, Frank Richards (Professor, emeritus, Yale University, New Haven, CT), for example, popularized a strategy that was eventually termed paratransgenesis. This is genetic transformation of a microbe carried by a host rather than the host and became a new way of delivering a gene product to control a pathogen.

We now call this approach "symbiotic control" to put the emphasis on the control strategy instead of the genetic transformation tactic. Symbiotic control is based on a natural phenomenon, as natural, as Frank suggests, as yoghurt, in which "friendly" bacteria displace "unfriendly" bacteria in a competitive displacement paradigm (some authors call this strategy "probiotics," others "replacement therapy"). This is a natural phenomenon because all animals depend on it to repel pathogenic invaders. Also coprophagy is very common in nature to allow re-digestion of a given meal, but also to ensure that a population of helpful symbionts is maintained in the gut.

6.2 Pierce's Disease

Pierce's disease (PD) was originally called Anaheim or Orange County disease before Pierce (1892) described the pathogen because of the severe manifestations in the region of coastal California just south of Los Angeles. It was acknowledged

early on that European grapevines could not be grown in Orange County because of a predilection for the disease to occur there. Thus the disease and therefore the pathogen were known from historic records in the nineteenth century in California. Before 1990, PD also occurred occasionally in northern California, especially Napa and Sonoma wine-growing regions.

The endophytic microbe, *Xylella fastidiosa*, was eventually identified as the pathogen causing PD. It is part of a complex including individual strains responsible for specific scorch diseases in a number of host plants (Redak et al. 2004) and is thought to have evolved in the new world from the region bordering the Gulf of Mexico as exclusively xylem-limited (Scally et al. 2005; Schuenzel et al. 2005). It is transmitted between host plants by xylem-feeding insects.

Prevalence of PD in California changed in the late 1990s when a new vector insect (*Homalodisca coagulata*, later *H. vitripennis;* Takiya et al. 2006) known as the glassy-winged sharpshooter (GWSS) moved into southern California (Blua et al. 1999) and eventually caused an epidemic in the Temecula, CA wine-growing region of southern Riverside, County. Although the route and exact date of entry of the vector insect are lost to obscurity, it hardly matters compared to the stark reality of the impact, which was described anecdotally in the following way. The first year, 3 vines were affected, the second year 100 vines were affected, and the third year 10,000 vines were affected (in one particular vineyard in Temecula, CA).

Much sensational and exaggerated reporting came from that 1996–1999 epidemic including predictions of the destruction of all vineyards in Temecula. Indeed around one third of the acreage of vineyards was lost before control measures took effect and the verbiage cooled off. Moreover, the severity of the epidemic was not uniform. Rather than killing every grapevine variety, the initial epidemic left a capricious pattern with 100% of some varieties lost while across the street a neighboring vineyard with the same variety suffered only 13% loss. No one can explain this pattern. One organic vineyard of old Petit Shirah grapevines (Bella Vista Winery, Temecula, CA) did not show symptoms of Pierce's disease while across the street Chardonnay grapevines were lost in the initial epidemic. This Petit Shirah tested positive by ELISA for a very small amount of *Xylella* bacteria, but only late in the season (Hill and Miller 2007). This apparent resistance to PD is not explained either.

By mid-2009, some 10 years later, the Temecula winegrowing region rebounded very well; several new wineries opened and new vineyards were and are being planted so that the acreage is approaching what it was before the epidemic started. The solution was fairly simple, a single treatment with the systemic insecticide imidacloprid by drip irrigation to the vineyards just ahead of the early summer/late spring appearance of GWSS combined with imidacloprid treatment of the main over-wintering host, citrus, in the immediate vicinity (Perring et al. 2001).

While this initially cost $200.00 an acre, patent protection for imidacloprid has since expired and generic substitutes cost far less, $79.00 an acre (Robert Wynn, California Department of Food and Agriculture, May 1, 2009, public lecture in Temecula). Today, the greatest threat to vineyards in the Temecula area is complacency. The insect and the pathogen are both present, sometimes in organic crops of citrus and grapevines that cannot be treated with systemic insecticides, or in abandoned

vineyards overrun by weeds, but still capable of harboring the pathogen while vineyards treated with imidacloprid have not shown losses other than to rodents and are back to replacement rates of grapevines that existed before the epidemic occurred.

This, then, is the maintenance level. It is widely held that if either systemic insecticide treatments stop or if GWSS develops resistance to the main systemic insecticide used, Pierce's disease will resurge. Despite outstanding efforts in biological control, predators and parasites have not had a significant impact on GWSS populations in California. Efforts continue to try to find a natural control combination that might ease need for insecticides. One such successful example of biological control was documented for a GWSS invasion of Tahiti (Grandgirard et al. 2008). However, the reason why such spectacular population crashes following parasite introductions can occur in one place (French Polynesia), but not another (California) remains unexplained.

6.2.1 Fastidious Insects and Bacteria

Xylella fastidiosa is so-named because it is slow-growing in culture (it is fastidious) (Mizell et al. 2008). In Riverside, CA in the middle of the quarantine zone, which encompasses most of southern California, GWSS are usually present all year, but are much more abundant starting at the end of May. When GWSS are collected by sweep net, brought into the laboratory and placed in cages on host plants, they start to die in a few days and the population can be completely dead in 2 weeks. This longevity depends to a certain extent on what time of year the collection was made. Without further precautions, experiments must be conducted within this period of longevity before mortality overcomes the experimental population.

A single literature reference specifically describing the difficulty of rearing GWSS is not known. However, colleagues report privately that the presence of at least three host plants in rearing cages is important to maintain populations longer that a few weeks. Each laboratory seems to have worked out its own protocols. The rearing is described as not so much difficult as tedious (Almeida, May 2009, personal communication; Leopold, May 2009, personal communication; Leopold 2007).

6.2.2 Transmission of Pathogen by GWSS

Purcell and Hopkins (1996) reported that only xylem-feeding insects are vectors of *Xylella*, some are more efficient at transmission than others; there is a very short latent period between acquisition and infectivity; and infectivity is lost after molting. This, plus images of bacteria lining the buccal cavity of vector insects has reinforced the widely held assumption that *Xylella* is carried by vector insects attached to the cuticle of the oral cavity. Since the foregut of insects is an extension of the integument and the cuticle lining is removed and replaced at each molt, and since reports claim that all contaminating bacteria are lost at each molt (Lopes et al. 2009), the conclusion is that *Xylella* are attached to the cuticle of the oral cavity or foregut.

Attachment of the pathogen, *Xylella*, to the cuticle of GWSS mouthparts (Backus 1985) appears to be a complex process (Killiny and Almeida 2009), but since the original report of Purcell et al. (1979), the transmission of *Xylella fastidiosa* is widely accepted as involving only attachment to the buccal cavity and physical movement between vector insects and host plant xylem. According to Killiny and Almeida (2009), for example:

> *X. fastidiosa* cells have been shown to colonize specific areas of the foreguts of insects, where they multiply and form a carpet-like biofilm (Purcell et al. 1979) … very dilute sap nutrients, passing through the foregut at 5 to 50 cm/s, being ingested by the insects. This turbulent environment is expected to cause occasional detachment of cells prior to the formation of mature biofilms within vectors (see Almeida et al. 2005, for a discussion of this topic).
>
> Very few bacteria in the head are sufficient for efficient transmission, implying that the key location in the foregut from which the bacteria are transmitted is very small … the precibarial valve blocks back-flow of fluid from the cibarium during ingestion as the cibarial diaphragm closes … (Purcell, Spring 2009, personal communication).
>
> … 'fluttering' (or the term Elaine Backus uses…) of the precibarial valve during xylem vessel penetration and prior to ingestion may be important and associated with *Xf* inoculation into plants, in addition to the cibarial muscles relaxing and creating turbulence. (Almeida, Spring 2009, personal communication).

Thus the widely held view is that bacteria detach from the turbulent flow in the buccal cavity (and are presumably carried into the plant during feeding). Since the movement of plant sap, as indicated above, is exclusively into the foregut through food channels connected to the xylem vessels during feeding, the acquisition of endophytic bacteria from the xylem is clear and logical; however, the transmission of bacteria from vector to plant host must occur during initial probing. The only other possibility is during periods when the cibarial pump muscles relax and the lower pressure of the xylem vessels pulls fluid into the plant from the lumen of the foregut via the food channel of the vector insect's stylets. However, there is a second possibility.

Jose Ramirez discovered that *Xylella fastidiosa* was present in the actively extruded salivary fluid of GWSS (Ramirez et al. 2008a, b). GWSS exhibits the unusual behavior of attempting to feed from any surface including plastic Petri dishes. These feeding attempts include secretions of salivary fluids. Presumably there is no movement of fluid through the food channels in the stylets during these feeding attempts.

> The results of the Ramirez paper are certainly provocative, and I have thought about them a lot ever since their publication. I refer to them in my X wave paper [Backus et al. 2009]. It is important that only 40% of the insects (10/25) that were observed to salivate on the tube walls had *Xf* in their saliva; 60% had none. And, as Ramirez admits, there was no correlation between the titer of *Xf* in the heads and the frequency of detection of *Xf* in saliva. As mentioned above, Alhaddad's findings [Alhaddad et al. 2011] show that salivation occurs readily with physical manipulation of the labium. These data strongly suggest to me that, 60% of the time, salivation during pre-penetration labial exploration is direct secretion from the salivary glands, and 40% of the time, may include some egestate from the precibarium (which may or may not dislodge a few bacteria)
>
> (Email 8 June 2009, Elaine Backus).

6.2.3 *Alcaligenes xylosoxidans var. denitrificans (Axd)*

GWSS were obtained from citrus trees in experimental citrus orchards at UC Riverside and surface sterilized. Stabs from contents of the heads were streaked on culture plates and the resulting colonies identified. Among these was *Alcaligenes xylosoxidans* var. *denitrificans* (Axd) that was named on the basis of response to culture conditions, and appearance and response to simple biochemical tests (Bextine et al. 2005a). Axd was genetically modified to carry a red fluorescent protein gene, DsRed and injected into six potential host plants. Samples were taken 2 weeks later 5 cm above the injection site and analyzed by quantitative RT-PCR.

The largest colony of Axd was found in lemon, *Citrus limon*. The remaining seedlings contained decreasingly smaller colonies in the order: *Citrus sinensis* (sweet orange) > *chrysanthemum grandiflora* cv. White Diamond > *Vinca rosea* (periwinkle) > *Lagerstroemia indic*a (crepe myrtle) > *Vitis vinifera* cv. Chardonnay, (grapevine). These data reflected the host preferences for the endophytic bacterium.

Because Axd occupied the same site in the vector insect as the pathogen (Bextine et al. 2004), and because both were xylem-limited endophytes in various host plants, Axd was seen as a viable delivery vehicle for a symbiotic control strategy. We inquired about the regulatory procedures this endophyte might entail.

Robert I. Rose, formerly of BRS (Biotechnology Regulatory Service) USDA-APHIS (United States Department of Agriculture-Animal Plant Health Inspection Service) did a review of the Genus, *Alcaligenes* and sent a summary of what he found including "may cause opportunistic infections" (e-mail, 4 February 2002). He also determined that species of *Alcaligenes* were "… apparently neither plant pathogens, nor livestock and poultry pathogens of any significance, thus probably not subject to APHIS PPQ [Plant Pest Quarantine] or VS interstate shipment permitting requirements."

6.2.4 *Identity of Axd*

Five symbionts isolated from the heads of surface-sterilized glassy-winged sharpshooter (GWSS) were identified through biochemical testing as *Alcaligenes xylosoxidans denitrificans* (*Axd*) Hc01, *Axd*1, *Axd*2, *Axd*3, and *Axd*4. The genetic relatedness of these bacteria, as well as their relationships to other bacterial species (Table 6.1), was analyzed. In order to avoid any possible distortions that could occur due to the random nature of base substitutions or possible gene transfer events in the phylogenetic trees (Yamamoto et al. 2000), the species were placed using two conserved genes, namely those encoding for the 16S ribosomal subunit and the gyrase B protein. Both of these genes are known to be highly conserved and universally present, and they are commonly used for constructing phylogenetic trees (Laguerre et al. 1994; Yamamoto and Harayama 1995).

Table 6.1 Bacterial strains used to construct the phylogenetic trees and their sources

Bacterial species	Source	Abbreviation used in trees
Achromobacter denitrificans ATCC 13138	American Type Culture Collection	AD13138
Achromobacter denitrificans ATCC 15173	American Type Culture Collection	AD15173
Achromobacter denitrificans LMG 1231	University of Michigan, Ann Arbor	AD1231
Achromobacter piechaudii LMG 1873	University of Michigan, Ann Arbor	AP1873
Achromobacter ruhlandii LMG 1866	University of Michigan, Ann Arbor	AR1866
Alcaligenes faecalis 16.7	California State University, Hayward	AF16.7
Alcaligenes faecalis LMG 1229	University of Michigan, Ann Arbor	AF1229
Alcaligenes odorans 10.7	California State University, Hayward	AO10.7
Alcaligenes xylosoxidans denitrificans 1	California State University, Hayward	Axd1
Alcaligenes xylosoxidans denitrificans 2	California State University, Hayward	Axd2
Alcaligenes xylosoxidans denitrificans 3	California State University, Hayward	Axd3
Alcaligenes xylosoxidans denitrificans 4	California State University, Hayward	Axd4
Alcaligenes xylosoxidans denitrificans Hc01	California State University, Hayward	rAxd
Burkholderia cepacia 6.7	California State University, Hayward	BC6.7
Pseudomonas aeruginosa 1.7	California State University, Hayward	PA1.7
Pseudomonas aeruginosa 11.7	California State University, Hayward	PA11.7
Pseudomonas aeruginosa 13.7	California State University, Hayward	PA13.7
Pseudomonas aeruginosa 2.7	California State University, Hayward	PA2.7
Pseudomonas aeruginosa 7.7	California State University, Hayward	PA7.7
Pseudomonas aeruginosa ATCC 10145	University of California, Riverside	PA10145
Pseudomonas fluorescens 3.7	California State University, Hayward	PF3.7
Pseudomonas pseudoalcaligenes 8.7	California State University, Hayward	Pps8.7
Pseudomonas pseudoalcaligenes ATCC 17440	American Type Culture Collection	Pps17440
Pseudomonas putida 4.7	California State University, Hayward	PP4.7
Pseudomonas putida 5.7	California State University, Hayward	PP5.7
Pseudomonas stutzeri 14.7	California State University, Hayward	PS14.7
Sheulamella putrefaciens 15.7	California State University, Hayward	SP15.7
Stenotrophomonas maltophilia 12.7	California State University, Hayward	SM12.7
Xanthomonas maltophilia 9.7	California State University, Hayward	XM9.7

The 16S tree in Fig. 6.1 shows that *Axd* Hc01 did not group with any of the other *Alcaligenes* or *Achromobacter* genera that were included in this study. Instead, it grouped most closely with a member of the genus *Pseudomonas*. Further analysis using gyrase B gene sequences (Fig. 6.2) also indicated that *Axd* Hc01 did not group with any of the *Alcaligenes* or *Achromobacter* species that were included in the study.

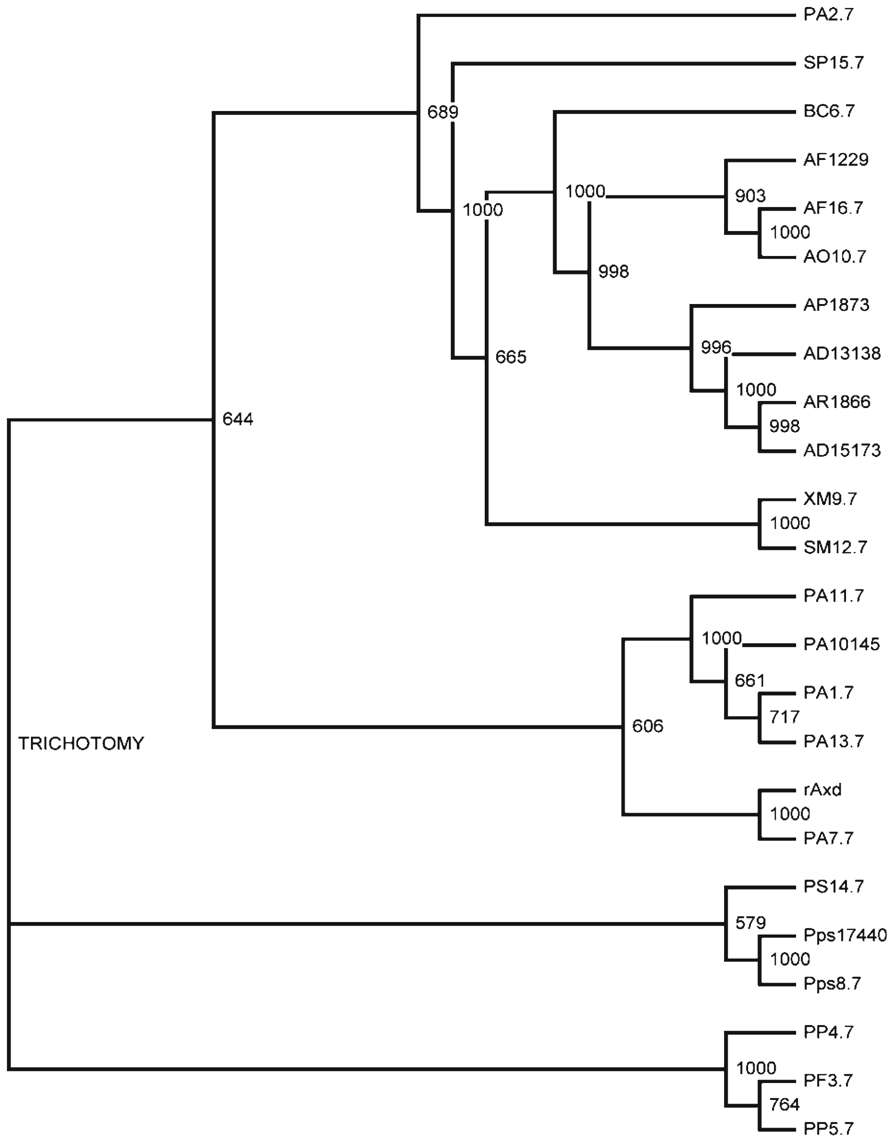

Fig. 6.1 16S tree with r*Axd* (*Axd* Hc01)

6.3 Mistake in Approaching the Regulatory Process

As shown in Figs. 6.1 and 6.2, the initial identification of *Alcaligenes*, based on biochemical characteristics and culture responses, was incorrect. Indeed, Blake Bextine (my postdoctoral fellow at the time) was certain that the bacterium we identified would eventually require a new name. Because this phylogenetic study

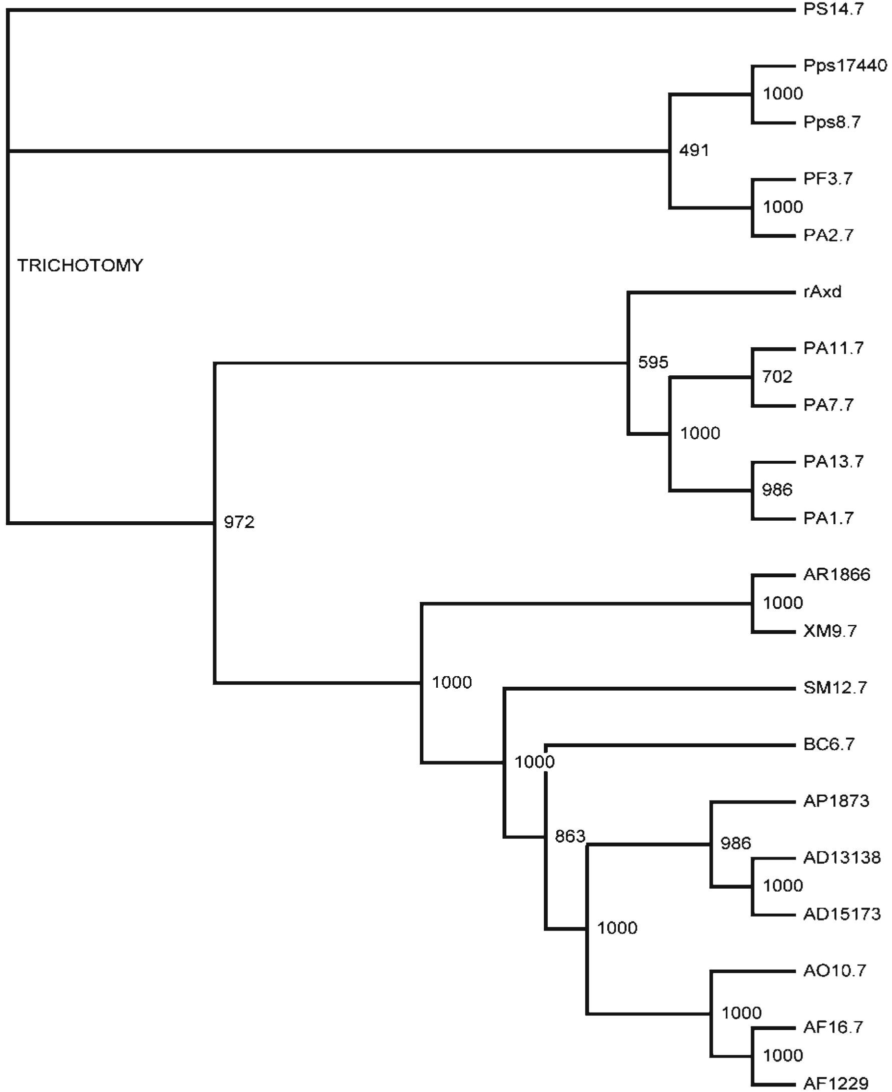

Fig. 6.2 Gyrase B tree with r*Axd* (*Axd* Hc01)

was completed during and after field trials were done, submitting the incorrect name *Alcaligenes* was a mistake because regulators had no choice but to rule based on the assumed identity of the organism.

Regulatory officers have nothing to go on besides the name of the organism submitted. A review of the literature (such as done by Bob Rose, mentioned above) using the name we provided revealed a connection between the Genus *Alcaligenes* and nosocomial infections in patients with cystic fibrosis (CF) and those with compromised

immune systems. It stands to reason that most microbes in the Gene Bank would be those generating research funds, which would be biased toward pathogens and nosocomial bacteria.

We speculated that bacteria selected to colonize the xylem fluid of plants and the oral cavity of sharpshooter insects were poor candidates for colonizing the lung tissue of humans. However, this speculation alone reveals yet another side-track.

We diverted further from the aims of the project by speculating about experiments needed to show that the "*Alcaligenes*" we isolated could not colonize the lungs of cystic fibrosis patients. Such work was better conducted in laboratories with cystic fibrosis infection models. Indeed, moving in this direction traps the researcher into trying to prove a negative and detracts from the substance of the project.

6.3.1 Rationale for Field Trials

We wanted to know the behavior of Axd in the xylem fluid of plants to understand the longevity of the candidate for symbiotic control. While Axd was in the correct location occupying the same niche as the pathogen, we did not know how successfully Axd would colonize grapevines in a vineyard. Moreover, it was important to conduct trials in grapevines in commercial vineyards.

Why couldn't you do this in the laboratory? Laboratory results are not necessarily accurate predictors of behavior in the field. Indeed, our original results from laboratory studies did reveal that Axd preferred citrus to the other host plants tested, especially grapevines (described above and in Bextine et al. 2004). When Axd was initially identified, the vector insects were collected from extensive experimental citrus orchards maintained at UC Riverside. They were surface sterilized and the colonies of bacteria obtained from the heads.

While this procedure made perfect sense; the symbiont had to be associated with the insect, we did not fully appreciate at the time that we were biased towards finding a symbiont associated with citrus instead of grapevines, which turned out to be the case. If we wanted a xylem-limited endophyte biased toward grapevines, we should have been sampling GWSS feeding on grapevines; and this lead to the next logical question of which grapevine variety to use as a source of symbionts from GWSS. Presumably each variety of grapevines has its own particular xylem compliment of symbionts. If a citrus-biased symbiont were used to deliver an anti-disease strategy, it could intercept the vector insect traveling from citrus to grapevines. For these reasons, determining the field behavior of the delivery vehicle symbiont seemed especially important.

6.3.2 Interstate Shipment – Beginning the Regulatory Process

The research project we had was funded by the Pierce's disease program of USDA-APHIS and the director at the time, Lloyd Wendel, asked about any regulatory hurdles that might hinder progress. By this time, our collaborator Dave Lampe had already

genetically marked our Axd with the DsRed gene (Bextine et al. 2005b). Since Axd "fell between the cracks" so to say, not being a pathogen or plant or animal pest, no permits were required for interstate shipment. The marked Axd was promptly sent from the Lampe lab at Duquesne University in Pittsburgh to UC Riverside to start laboratory tests. These were done under the auspices of the Institutional BioSafety Committee at UC Riverside. BSCs are required to review and approve research on all transgenic organisms at institutions receiving federal funding.

It was subsequently also determined (by USDA-APHIS-BRS; United States Department of Agriculture-Animal Plant Health Inspection Service-Biotechnology Regulatory Service) that genetically altered Axd fell outside the regulatory arena of BRS. Luckily, Dr. Rose, who determined this, had previously worked for the Environmental Protection Agency (EPA) and had many contacts there.

The first and most helpful was William Schneider, who warned about the regulatory experience of a bacterium called *Burkholderia cepacia* that was proposed for use as a plant protection biopesticide (Holmes et al. 1998). Because *Burkholderia* was described as having a predilection for colonizing lungs in patients with cystic fibrosis and was called an opportunistic human pathogen in immuno-compromised patients (Holmes et al. 1998). It was classified as a nosocomial bacterium and was eventually withdrawn from the registration procedure.

At this point the project split into separate parts dictated by the regulatory realities and unfortunately diluting the main effort. The first part was to sequence parts of the genome of what we were calling Axd to better define it in hopes that it was actually an innocuous bacterium that could be more favorably viewed by EPA and not suffer the fate of *Burkholderia*. Another effort was aimed at testing the fate of genetically marked Axd in field trials. The rest of the project continued searching for gene products and perfecting their secretion by the vehicle endophyte.

6.3.3 Field Trials

Guided by advice from Bob Rose and Bill Schneider, permits were sought to determine how the genetically marked endophyte behaved in grapevines in commercial vineyards. Genetically marked Axd was not a biopesticide. Had it contained an anti-*Xylella* factor, it would have been so designated and would have fallen under a different regulatory division of EPA. Instead by internal agreement, a transgenic organism such as transgenic Axd was given to TSCA for regulation (Toxic Substance Control Act).

Email from Robert Rose 6 January 2003

> Your situation is a bit complex from the regulatory perspective.
>
> … an agent that produces a gene product that is pesticidal in effect to *Xylella fastidiosa* that causes Pierce's disease of grapes (or does it merely displace *Xylella* as an endophyte in the plant?), particularly a serious problem of grapes in CA. That would require an Experimental Use Permit from EPA, Biopesticides & Pollutions Prevention Division: http://www.epa.gov/pesticides/biopesticides/.

Table 6.2 Outline of TSCA experimental release application

A. Recipient organism
B. Subject organism characterization
C. Potential human health effects of subject microorganism
D. Ecological effects
E. Predicted production volume, byproducts, use and consumer exposure
F. Predicted releases due to manufacturing of subject microorganism
G. Information applicable to field tests of subject microorganism
References cited

Email from William Schneider 7 January 2003

It appears that at the moment, with just marker genes, you would not need to submit a biotech Notification to us prior to testing these in the field since it doesn't have any pesticidal characteristics yet. However, as soon as you add a gene to neutralize the *Xylella fastidiosa* pathogen, that will make it a pesticide and it will be subject to regulation with us.

If it turns out that this (without pesticidal genes) is not regulated by another federal agency, it is very likely that it will fall under the authority of TSCA, that is, the Toxic Substances Control Act, as handled by the Office of Pollution Prevention and Toxics of EPA (OPPT). You can find information at http://www.epa.gov/opptint/biotech/. You can ask Gwen McClung about this (cc'ed above).

First contact was with James Alwood, Biotechnology Coordinator, Chemical Control Division. We sent in the first application to him May 1, 2003. It contained a detailed description of the "Recipient Organism," Axd; description of inserted genes; method of insertion; test sites; rationale for the genetic transformation; potential human health effects of Axd; insertion and method of insertion, any pathogenicity or infectivity. The later part of the description covered literature examples of reports of nosocomial infections. The application had Toxicity and immunological effects of Axd, if any; ecological effects of Axd; any data on environmental fate of Axd (which of course was impossible since this is what the permit was requested to determine).

This application ran to 30 pages single spaced and was like writing a grant application. The outline of the application is shown in Table 6.2. In the end one graduate student was assigned to put the application together, but it required information from all collaborators including Carol Lauzon at California State University East Bay and David Lampe at Duquesne University.

The information contained in this application is interesting. It is a shame it is not published. Some applicants do not want information made public because of proprietary information they contain. Ours was not one of these and the application could and probably should have been posted online for transparency; however the agency has no method for doing this. We also learned later that any changes in the websites are very difficult to make and take months if not years. Professionals working in EPA find this frustrating at times. [See the executive summary at http://biopesticide.ucr.edu]

The initial application was sent as mentioned in 1 May 2003. Approval of the application came 2 July 2003. The letter of notification included:

Re: TERA R-03-01

You may proceed with the field tests described in the TERA until December 31, 2003, however, at the conclusion of the field test, you must destroy all test plants and treat or excavate the soil surrounding the plants according to methods approved by EPA.

EPA has determined, pursuant to 40 CFR 725.270(b)(2), that the research and development activity for this microorganism as described in the TERA application, does not present an unreasonable risk of injury to health or the environment. However, in the event of a future submission of this microorganism for commercial use in a microbial commercial activity notice (MCAN), the following issues will need to be addressed. These issues have generally been a concern for EPA in its evaluation of environmental releases of other intergeneric [sic] microorganism:

(1) Any data on the survival of the microorganism when released to the environment.
(2) A more detailed documentation of the construct to provide evidence the genetic material has been stably inserted. You will conduct experiments on the microorganism to see if the insertion is chromosomally located or it is carried on plasmids.

EPA would be interested in reviewing that data as the data from both of these studies would help EPA to better evaluate TERAs and MCANs such as yours.

Determining details of the insertion of the DsRed marker gene into Axd was not part of our original research plans. It was understandable that the genetic makeup of the final product would be of interest, but it is not clear what use that would be. A series of nucleotide sequences showing the inserted marker gene sequence nested into the chromosomal DNA of the host Axd would not provide as much stability or longevity information as rearing the symbiont through several generations under different conditions.

This request was a reminder that EPA is designed to deal with companies who had commercial development of products in mind and had sufficient funding for studies in support of regulation. This was information that would be needed for a final use permit. EPA could have suggested funding this work through one of the federal risk assessment grant programs in view of the very last paragraph. EPA should look at applications lacking proprietary information from research universities as opportunities to foster new technology by offering to suggest funding sources for the work requested in items (1) and (2) in the letter above.

EPA representatives explained privately (as opposed to officially) that a Scientific Advisory Panel took up the issue of this application and ruled that heavy restrictions should be applied ("… destroy all test plants…"). The main reason apparently came from cystic fibrosis concerns, as mentioned above. EPA and the advisory panel were not required to explain their ruling although it is difficult for the average person to understand how microorganisms used to treat grapevines in commercial vineyards in a small trial had anything to do with immuno-compromised or CF patients.

Indeed, the SAPs are very much like peer reviewers cloaked in a collective anonymity. In this position they are able to squash development of promising new technology with impunity; much like grant reviewers. EPA should find ways to alleviate this perception. The "destroy all the plants" order made it impossible to determine if Axd would make it from one season to another in a treated plant. In the end this was unnecessary.

6.4 Lack of Longevity of RAxd in Grapevines in Vineyards

Table 6.3 shows the isolation of RAxd from mature grapevines in three locations in California, near Temecula in Riverside County, near Bakersfield in Kern County and near Napa in Napa County. RAxd was injected into the trunks of mature grapevines (Needle), misted onto the leaves (Foliar) and applied as a soil drench (Soil). As shown in the Bakersfield test, 2 weeks post-inoculation, RAxd was found in leaf petiole samples. However, after 4 weeks samples declined and by 6 weeks no RAxd was detected.

At the end of the trial before materials were destroyed (Fig. 6.4), no RAxd was detected in berries, canes or roots of the treated plants. We took this as confirmation that Axd and RAxd were not natural endophytes of these varieties of grapevines in these locations. Moreover, the Temecula trials revealed no positive identifications of the introduced microbes. We attributed that to differences in different varieties of grapevines as well as possibly location effects. The bottom line was RAxd was ineffective as a treatment of grapevines in commercial vineyards if it was expected to be present to guard against introduction of Xf by vector insects (Table 6.3 and Fig. 6.3).

Table 6.3 Presence or absence of RAxd in grapevines follow treatment of grapevines in commercial vineyards (From Miller 2007)

	Axd positive samples[a]						
	Weeks post-inoculation				During grapevine removal		
Application method	0	2	4	6	Berries	Canes	Roots
Bakersfield							
Foliar	0	3	2	ND	0	0	0
Needle	0	2	0	ND	0	0	0
Soil	0	3	0	ND	0	0	0
Control	0	0	0	ND	0	0	ND
Napa							
Foliar	0	0	0	0	0	0	0
Needle	0	0	0	0	0	0	0
Soil	0	1	1	0	0	0	0
Control	0	0	0	0	0	0	ND
Temecula							
Foliar	0	0	0	0	0	0	0
Needle	0	0	0	0	0	0	0
Soil	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	ND

Foliar leaf spray, *Needle* inoculation of trunk, *Soil* soil drench, *Control* no treatment, *ND* not determined

[a] Six samples from five grapevines per treatment per site (n = 30 plants per grapevine)

Fig. 6.3 Grapevine in Napa, CA summer 2004, inoculated with RAxd and covered with gauze to prevent access by xylem-feeding insects. These vines were volunteered for this test by John G Williams because they were slated for routine replacement

6.5 Alternative Symbiotic Control Agents

A survey of other bacteria isolated from GWSS foreguts was conducted and these were identified using 16S rDNA probes. The resulting sequences were then run through BLAST so that they could be placed in the correct genus. Bacteria belonging to the genus *Methylobacterium* were most frequently found. In addition, some of the bacteria belonged to *Bacillus*, *Cryocola*, and *Pedobacter*.

Various symbionts isolated from the plants have been reported to provide resistance to plants against biotic as well as abiotic stresses. In particular, *Paenibacillus polymyxa* was reported to confer resistance to drought and resistance to *Erwinia carotovora* infection in the model plant species *Arabidopsis thaliana* (Timmusk and Wagner 1999). Our lab isolated a member of the genus *Paenibacillus* from a population of apparently PD-resistant grapevines located in the Weaver Vineyard in the Temecula, California region and also from Agricultural Operations UC, Riverside (Fig. 6.4).

This bacterium was reported to provide resistance against water stress. *Xylella fastidiosa* (Xf) infection also results in water stress like symptoms. Infection of grapevines with Xf first results in parts of the leaves drying out and turning brown. Adjacent tissues turn yellow or red at this time. Slowly, desiccation spreads and the leaves may shrivel and drop, with only the petioles remaining attached to the stem.

Fig. 6.4 Grapevines being burned at a commercial vineyard near Bakersfield, CA in December 2004 in accordance with permit requirements

Diseased stems mature irregularly, with patches of brown and green tissue. In addition, the diseased plants produce stunted chlorotic shoots. Infected plants rarely survive more than 2 years (Hopkins 1989). Since the symptoms of water stress and *X. fastidiosa* infection symptoms are so similar, we hypothesized that *Paenibacillus* sp. (PB) might help in either masking or delaying the onset of PD symptoms, or even provide resistance against *X. fastidiosa* infection. The results of our preliminary experiments indicated that our isolate of *Paenibacillus* was capable of providing resistance against the effects of water stress in grapevines (Fig. 6.5).

I am excited to hear that we will officially kick off the project soon! Arinder is also very eager to participate. We are currently looking at an alternative symbiont, Pantoea agglomerans, for performing our preliminary work. Axd must be inoculated into the plant and appears to have limited movement within the plant. However Arinder has recently found that Pantoea, when sprayed on the plant, is taken up throughout the whole plant. He has also recently found that the insect can acquire Pantoea from the sprayed plant and has preliminary evidence that the insect can subsequently transmit the bacteria. It is our idea to use this symbiont to test the delivery system and determine later whether we will continue the project using Pantoea or if we will also test with Axd. Please let us know if you have any immediate concerns with this approach (Ravi Durvasula, Personal communication).

Fig. 6.5 (**a**) Plant kept under water stress. (**b**) Plant inoculated with *Paenibacillus* species and kept under water stress

6.6 Conclusion

Whichever method proves successful in protecting grapevines from PD, the candidate symbiotic control agent must lack side effects such as the ability to infect lungs of cystic fibrosis patients, must survive in grapevines over winter or re-inoculated on a season basis and retain the ability to displace pathogenic strains of Xf. The regulatory process should be engaged early.

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References

Alhaddad H, Coudron TA, Backus EA, Schreiber F (2011) Comparative behavioral and protein study of salivary secretions in *Homalodisca* spp. sharpshooters (Hemiptera: Cicadellidae: Cicadellinae). Annals Entomol Soc Am 104:543–552

Almeida RPP, Blua MJ, Lopes JRS, Purcell AH (2005) Vector transmission of *Xylella fastidiosa*: applying fundamental knowledge to generate disease management strategies. Ann Entomol Soc Am 98:775–786

Backus EA (1985) Anatomical and sensory mechanisms of leafhopper and planthopper feeding behavior. In: Nault LR, Rodriguez JG (eds) The leafhoppers and planthoppers. Wiley, New York, pp 163–194

Backus EA, Holmes W, Schreiber F, Reardon BJ, Walker GP (2009) Sharpshooter X wave: correlation of an EPG waveform with xylem penetration supports a hypothesized mechanism for *Xylella fastidiosa* inoculation. Ann Entomol Soc Am 102:847–867

Bextine BR, Harshman D, Johnson MC, Miller TA (2004) Impact of pymetrozine on glassy-winged sharpshooter feeding behavior and rate of *Xylella fastidosa* transmission. J Insect Sci. http:.//www.insectscience.org/4.34/

Bextine B, Lampe D, Lauzon C, Jackson B, Miller TA (2005a) Establishment of a genetically marked insect-derived symbiont in multiple host plant. Curr Microbiol 50:1–7

Bextine B, Blua M, Harshman D, Miller TA (2005b) A SYBR green-based real-time polymerase chain reaction protocol and novel DNA extraction technique to detect *Xylella fastidiosa* in *Homalodisca coagulata*. J Econ Entomol 98:667–672

Blua MJ, Phillips PA, Redak RA (1999) A new sharpshooter threatens both crops and ornamentals. Calif Agric 53:22–25

Enserink M (2009) Swine flu names evolving faster than swine flu itself. Science 324:871

Grandgirard J, Hoddle MS, Petit JA, Roderick GK, Davies N (2008) Engineering an invasion: classical biological control of the glassy-winged sharpshooter, *Homalodisca vitripennis*, by the egg parasitoid *Gonatocerus ashmeadi* in Tahiti and Moorea, French Polynesia. Biol Invasions 10:135–148

Hill BL, Miller TA (2007) Seasonal appearance of *Xylella* in vineyard and control of Pierce's disease in Southern California. In: Proceedings of the Pierce's disease research symposium, Westin Horton Plaza, San Diego, 12–14 Dec 2007, pp 200–201

Holmes A, Govan J, Goldstein R (1998) Agricultural use of *Burkholderia* (*Pseudomondas*) *cepacia*: a threat to human health? Emerg Infect Dis 4:221–227

Hopkins DL (1989) *Xylella fastidiosa*: xylem limited bacterial pathogen of plants. Annu Rev Phytopathol 27:271–290

Killiny N, Almeida RPP (2009) *Xylella fastidiosa* afimbrial adhesins mediate cell transmission to plant by leafhopper vectors. Appl Environ Microbiol 75:521–528

Laguerre G, Rigottier-Gois L, Lemanceau P (1994) Fluorescent *Pseudomonas* species categorized by using Polymerase Chain Reaction (PCR)/restriction fragment analysis of 16S rDNA. Mol Ecol 3:479–487

Leopold RA (2007) Colony maintenance and mass-rearing: using cold storage technology for extending the shelf-life of insects. In: Vreysen MJB, Robinson AS, Hendrichs J (eds) Area-wide control of insects: from research to field implementation. Springer, Dordrecht, pp 149–162

Lopes JRS, Daugherty MP, Almeida RPP (2009) Context-dependent transmission of a generalist plant pathogen: host species and pathogen strain mediate insect vector competence. Entomol Exp Appl 131:216–224

Miller TA (2007) Applying insect transgenic technology: scientific and regulatory experiences. Entomol Res 37:67–75

Mizell RF, Andersen PC, Tipping C, Brodbeck B (2008) *Xylella fastidiosa* diseases and their leaf-hopper vectors. http://edis.ifas.ufl.edu/IN174

Perring TP, Farrar CA, Blua MJ (2001) Proximity to citrus influences Pierce's disease in Temecula Valley vineyards. Calif Agric 55:13–18

Pierce N (1892) The California vine disease. The United States Department of Agriculture, Division of Vegetable Pathology, Bull. No. 2

Purcell AH, Hopkins DL (1996) Fastidious xylem-limited bacterial plant pathogens. Annu Rev Phytopathol 34:131–151

Purcell AH, Finlay AH, Mclean DL (1979) Pierce's disease bacterium: mechanism of transmission by leafhopper vectors. Science 206:839–841

Ramirez JL, Lacava PT, Miller TA (2008a) Detection of *Xylella fastidiosa* in GWSS saliva. J Insect Sci 8:34, available online: insectscience.org/8.34

Ramirez JL, Perring TM, Miller TA (2008b) Fate of a genetically modified bacterium in the foregut of glassy-winged sharpshooter. J Econ Entomol 101:1519–1525

Redak RA, Purcell AH, Lopes JRS, Blua MJ, Mizell RF III, Andersen PC (2004) The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. Annu Rev Entomol 49:243–270

Scally M, Schuenzel EL, Stouthamer R, Nunney L (2005) A multilocus sequence type system for the plant pathogen *Xylella fastidiosa* and the relative contribution of recombination versus point mutation to clonal diversity. Appl Environ Microbiol 71:8491–8499

Schuenzel EL, Scally M, Stouthamer R, Nunney L (2005) A multigene phylogentic study of clonal diversity and divergence in North American strains of the plant pathogen *Xylella fastidiosa*. Appl Environ Microbiol 71:3832–3839

Takiya DM, McKamey SH, Cavichioli RR (2006) Fixation of the type-species of *Homalodisca* Stål as *H. vitripennis* (Germar), the oldest name for the glassy-winged sharpshooter (Hemiptera: Cicadellidae: Cicadellinae). Annu Entomol Soc Amer 99:648–655

Timmusk S, Wagner EGH (1999) The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: a possible connection between biotic and abiotic stress responses. Mol Plant Microbe Interact 12(11):951–9

Yamamoto S, Harayama S (1995) PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Appl Environ Microbiol 61:1104–1109

Yamamoto S, Kasai H, Arnold D, Jackson R, Vivian A, Harayama S (2000) Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. Microbiology 146:2385–2394

Chapter 7
Over a Decade of Crop Transgenes Out-of-Place

Norman C. Ellstrand

Abstract An early concern about genetically engineered crops was that transgenes might have undesirable consequences if they ended up in plants and places for which they were not intended. It has been over two decades since the first transgenic plants were environmentally released and over a decade since they have been commercialized. It is time to take stock of crop transgenes out of place. I have assembled a selected compilation of 22 incidents of in which crop transgenes were detected in living organisms (seeds or plants) in situations for which they were not intended. Contrary to initial concerns, crop transgenes have rarely introgressed into wild populations, but rather have often ended up in a different variety of the same species. Evolving regulatory policy should heed the large (and increasing) number of well-documented cases of transgenes out-of-place. In particular, it should treat the processed products of transgenic crops differently from living organisms, such as grain, that, if planted, are capable of multiplying their genes.

Keywords Adventitious presence • Biosafety • Coexistence • Contamination • Cross-pollination • Ferality • Transgene flow • Hybridization • Low level presence • LLP • Seed spillage

7.1 Introduction

One of the earliest concerns about genetically engineered crops was that transgenes might have undesirable consequences if they ended up in plants and places for which they were not intended. The initial worry focused largely on a single issue: spontaneous hybridization between a transgenic crop and a wild relative growing

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nearby might deliver a novel trait that would result in the evolution of a new plant pest problem (Colwell et al. 1985; National Research Council 1989). Goodman and Newell (1985) put it well: 'The sexual transfer of genes to a weedy species to create a more persistent weed is probably the greatest environmental risk of planting a new variety of crop species,' transgenic or not.

Concern about the unintended movement of crop genes long preceded the advent of genetically engineering. Unwanted genes can enter a seed stock long before it is commercialized. For example, immigrant gene flow by pollen from cross-compatible plants outside of breeders' selection plots ('pollen contamination', Kelly and George 1998) frustrates their gains under artificial selection. Cross-pollination is not the only potential route for genetic admixture. Care must be taken to prevent accidental mixing of seeds of different commercial varieties to maintain the relative varietal purity for the consumer ('seed contamination', Strayer 2002). To illustrate, a farmer intending to grow flax for fiber would be disappointed indeed to find that most of her plants were of an oilseed variety!

Because 100% genetic purity is difficult or impossible to obtain, thresholds of unintended genetic material have been standardized for different purposes. For example, the OECD Seed Scheme requires a minimum of 99.9% varietal purity for oilseed rape intended for human consumption for basic seed (seed used as basis for varietal seed increase) and reduces the requirement to 99.7% for certified seed, the purest seed population normally grown by commercial farmers (OECD 2010).

Generally, farmers and others associated with crops and their products anticipate and tolerate low levels of genetic mixing. For the most part, low levels of admixture do not cause substantial harm. But some exceptions are notable. Spontaneous hybridization between sugarbeet and the wild sea beet in Europe has lead to the evolution of the noxious weed beet that has resulted in over a billion dollars of losses to Europe's sugar industry (Ellstrand 2003a). Likewise, hybridization between wild coconut palm and the domesticated coconut palm has resulted in the extinction of the former (Ellstrand 2003a). Both examples happened long before the advent of genetically engineered crops.

The unintended occurrence of transgenes or a transgenic variety is increasingly characterized by the term 'adventitious presence', sometimes as an alternative to the term 'contamination,' perhaps because the latter carries negative connotations. 'Adventitious presence' is variously defined: simply: 'unintended incidence of something rather than the desired crop' (Kershen and McHughen 2005), generally: 'the unintentional presence and accidental commingling of trace amounts of "off types" of seed or grain in a parcel of seed or grain' (Demeke et al. 2006), or rather specifically: 'unintended, technically unavoidable presence of biotech material in an agricultural commodity used for food and, in some instances, other end use purposes' (Council for Agricultural Science and Technology 2007).

Since the first transgenic crops were commercialized in the mid 1990s, reports of transgenes out-of-place have steadily increased (Greenpeace International 2007). Whether or not they fit one of the definitions of adventitious presence, these reports have frequently attracted the attention of both the general and the scientific media (e.g., Ledford 2007). Interestingly, the same technologies that gave rise to engineered

genes have also given rise to extraordinarily sensitive techniques that can detect their presence. In particular, detection via the polymerase chain reaction can reliably identify a specific genotype at a frequency as low as 1 out of a 1,000 seeds (Demeke et al. 2006; but see Pineyro-Nelson et al. 2009). Of course, noteworthy cases of the unexpected presence of transgenes are typically verified by testing in second lab or by testing with a second method (McHughen 2006).

Transgenic plants that occur in unintended locations or transgenes in unintended plants are of scientific interest because they have shed light on how crop genes move and mix both by natural processes such as pollen dispersal as well as by anthropogenic processes such as seed spillage. Successful gene migration, whether by wind, water, or the hand of man, is gene flow, one of the evolutionary forces (Ellstrand 2003a). Because transgenes are novel, easy to detect, and have an unambiguous evolutionary origin, their movements can provide an unparalleled model for revealing how plant genes move.

7.2 Crop Transgenes Out of Place

Despite the varied interest in the significance of transgene 'contamination', 'adventitious presence', and dispersal, I am not aware of a scholarly effort that both inventories and reviews incidents of crop transgenes out of place that have occurred since their inception. Now, more than 35 years after the first concerns were voiced about the unintended movement of transgenes, it is time to take stock. Table 7.1 is a selected compilation of 22 incidents of transgenes out of place. I have chosen the better-studied examples, relying on the peer-reviewed literature when available. In particular, my focus is on living organisms, plants or seed intended for planting because such organisms provide opportunity for continued spread. Cases involving the unintended processed products of transgenic plants (for example, found in food) are not included (such as the notorious StarLink affair (Bucchini and Goldman 2002)). Likewise avoided are cases involving intended (but illegal) movement of smuggled transgenic seeds across borders, for example, transgenic soybean smuggled from Argentina into Brazil during the first few years of this century (Cohen and Paarlberg 2004). When possible and practical, each entry in the Table is assigned to a specific transgenic event. When in doubt, assignment is made to a deregulated transgenic event based on the phenotype of the plant (for example, unless other information is available, glyphosate-resistant *Brassica napus* canola in Canada is assigned to event GT73, the only deregulated event with that phenotype in that country).

Table 7.1 illustrates the variety of cases of transgenes out of place. In some cases, transgenes that have not yet been deregulated have been found in seed lots, in plants under production, or in plants growing wild in the field. In other cases, transgenes that have been deregulated in one country have been found in plants or seed in another country where they have yet to be deregulated.

Feral transgenic plants (with regulated or deregulated events), and their descendants have been found as free-living organisms in ruderal habitats. In the case of feral

Table 7.1 Examples of crop transgenes out of place

Date of initial discovery or initial report	Scientific and common name of source crop (Event name or names, if known)[a]	At the time of the discovery, transgenic plant's owner (Current owner)	Unintended destination of transgene ('seed' means 'intended for planting' as opposed to 'grain', which is intended for consumption or processing)	Transgenic phenotype[a]	At the time of discovery, was the transgenic crop … Deregulated?/ commercially available?	Relevant citation(s)
1997	*Brassica napus* Roundup Ready® canola (GT200)	Monsanto	Seed of extremely similar, commercialized, transgenic Monsanto variety	Glyphosate resistance via 5-enolpyruvylshikimate-3-phosphate synthase and glyphosate oxidoreductase	For environmental release only/No	Demeke et al. (2006)
1998	*Brassica napus* Roundup Ready® canola (GT73)	Monsanto	Volunteer canola plants in Canadian agricultural fields, including intervarietal hybrids with multiple transgenic events	Glyphosate resistance via 5-enolpyruvylshikimate-3-phosphate synthase and glyphosate oxidoreductase	Yes/Yes	Hall et al. (2000), Beckie et al. (2003)
1998	*Brassica napus* LibertyLink® canola (at least two different events)	Aventis CropScience (Bayer CropScience)	Volunteer canola plants in Canadian agricultural fields, including intervarietal hybrids with multiple transgenic events	Glufosinate resistance via phosphinothricin N-acetyltransferase	Yes/Yes	Hall et al. (2000), Beckie et al. (2003)
2000	*Brassica napus* LibertyLink® canola (insufficient information to assign)	Aventis CropScience (Bayer CropScience)	Various commercial canola seedlots in which the transgene was unexpected, including individual seed with multiple transgenic events in a transgenic Monsanto seedlot	Glufosinate resistance via phosphinothricin N-acetyltransferase	Yes/Yes	Beckie et al. (2003), Friesen et al. (2003)

2000	*Brassica napus* Roundup Ready® canola (GT73)	Monsanto	Various commercial canola seedlots in which the transgene was unexpected, including individual seed with multiple transgenic events in transgenic Aventis CropScience seedlots	Glyphosate resistance via 5-enolpyruvylshikimate-3-phosphate synthase and glyphosate oxidoreductase	Yes/Yes	Beckie et al. (2003), Friesen et al. (2003)
2001	*Brassica napus* Roundup Ready® canola (GT200)	Monsanto	Seed of extremely similar, commercialized, transgenic Monsanto variety	Glyphosate resistance via 5-enolpyruvylshikimate-3-phosphate synthase and glyphosate oxidoreductase	For environmental release only/No	Demeke et al. (2006)
2001	Maize (NK603 and at least one other)	Monsanto for NK603; insufficient information to assign the other(s)	Seed of maize landraces in Mexico (after a multiyear moratorium on transgenic maize planting)	Glyphosate resistance via 5-enolpyruvylshikimate-3-phosphate synthase and/or lepidopteran resistance via insecticidal Bt protein Cry1(Ab or Ac) (perhaps more)	Yes/Yes, (but not for environmental release in Mexico)	Quist and Chapela (2001), Ortiz-Garcia et al. (2005), Serratos-Hernández et al. 2007, Piñeyro-Nelson et al. (2009), Dyer et al. (2009)
2001	*Brassica napus* Roundup Ready® canola (GT73)	Monsanto	Spontaneous hybrids between wild birdsrape (*Brassica rapa)* and canola found in and near the crop – and their introgressed descendants	Glyphosate resistance via 5-enolpyruvylshikimate-3-phosphate synthase and glyphosate oxidoreductase	Yes/Yes	Warwick et al. (2003, 2008)

(continued)

Table 7.1 (continued)

Date of initial discovery or initial report	Scientific and common name of source crop (Event name or names, if known)[a]	At the time of the discovery, transgenic plant's owner (Current owner)	Unintended destination of transgene ('seed' means 'intended for planting' as opposed to 'grain', which is intended for consumption or processing)	Transgenic phenotype[a]	At the time of discovery, was the transgenic crop … Deregulated?/commercially available?	Relevant citation(s)
2003	*Lycopersicon esculentum* tomato (insufficient information to assign)	Petoseed/Zeneca Plant Science	Seed distributed to 12 U.S. institutions and to researchers in 14 other countries as a supposedly nontransgenic variety	Delayed softening via decrease of polygalacturonase level	Yes/No	University of California Davis (2003)
2003	*Brassica napus* Roundup Ready® canola (GT73)	Monsanto	Spontaneous canola plants in ruderal habitats in Japan, including intervarietal hybrids with multiple transgenic events	Glyphosate resistance via 5-enolpyruvylshikimate-3-phosphate synthase and glyphosate oxidoreductase	Yes/Yes	Saji et al. (2005), Aono et al. (2006), Nishizawa et al. (2009)
2003	*Brassica napus* LibertyLink® canola (insufficient information to assign)	Aventis CropScience (Bayer CropScience)	Spontaneous canola plants in ruderal habitats in Japan, including intervarietal hybrids with multiple transgenic events	Glufosinate resistance via phosphinothricin N-acetyltransferase	Yes/Yes	Saji et al. (2005), Aono et al. (2006), Nishizawa et al. (2009)
2004	*Carica papaya* papaya (55–1 or 63–1)	Cornell University	Hawai'ian papaya trees grown from supposedly non-transgenic seeds	Resistance to papaya ringspot virus via expression of virus coat protein	Yes/Yes	Manshardt et al. (2007)

2004	*Brassica napus* Roundup Ready® canola (GT73)	Monsanto	Spontaneous canola plants in ruderal habitats in Canada, including intervarietal hybrids with multiple transgenic events	Glyphosate resistance via 5-enolpyruvylshikimate-3-phosphate synthase and glyphosate oxidoreductase	Yes/Yes	Knispel et al. (2008)
2004	*Brassica napus* LibertyLink® canola (insufficient information to assign)	Aventis CropScience (Bayer CropScience)	Spontaneous canola plants in ruderal habitats in Canada, including intervarietal hybrids with multiple transgenic events	Glufosinate resistance via phosphinothricin N-acetyltransferase	Yes/Yes	Knispel et al. (2008)
2004	*Agrostis stolonifera* Roundup Ready® creeping bentgrass (ASR368)	Monsanto/Scott's	Spontaneous volunteers, spontaneous crop-wild hybrids, and their introgressed descendants in ruderal habitats in the United States	Glyphosate resistance via 5-enolpyruvylshikimate-3-phosphate synthase	No/No	Reichman et al. (2006, Reichman and Watrud 2007), Zapiola et al. (2008)
2005	*Zea mays* ssp. *mays* maize (Bt10)	Syngenta	Seed of another, extremely similar, commercialized, transgenic variety	Lepidopteran resistance via insecticidal Bt protein Cry1Ab and glufosinate resistance via phosphinothricin N-acetyltransferase	No/No	Demeke et al. (2006)
2006	*Oryza sativa* LibertyLink® rice (LLR601)	Bayer CropScience	Seed of the non-transgenic variety Cheniere	Glufosinate resistance via phosphinothricin N-acetyltransferase	No/No	USDA (2007a)
2007	*Oryza sativa* LibertyLink® rice (LLR604)	Bayer CropScience	Seed of the non-transgenic variety Clearfield 131	Glufosinate resistance via phosphinothricin N-acetyltransferase	No/No	USDA (2007a)

(continued)

Table 7.1 (continued)

Date of initial discovery or initial report	Scientific and common name of source crop (Event name or names, if known)[a]	At the time of the discovery, transgenic plant's owner (Current owner)	Unintended destination of transgene ('seed' means 'intended for planting' as opposed to 'grain', which is intended for consumption or processing)	Transgenic phenotype[a]	At the time of discovery, was the transgenic crop … Deregulated?/commercially available?	Relevant citation(s)
2007	*Zea mays* ssp. m*ays* maize (Event 32)	Dow AgroSciences	Seed of three hybrid lines that had not been commercialized	Coleopteran resistance via insecticidal Bt protein Cry34 and glufosinate resistance via phosphinothricin N-acetyltransferase	No/No	USDA (2008)
2009	*Linum usitatissimum* Triffid® flax (FP967)	University of Saskatchewan	Certified seed of supposedly non-transgenic varieties	Sulfonylurea herbicide tolerance via acetolactate synthase	Yes/No	Flax Council of Canada (2010)
2010	*Brassica napus* Roundup Ready® canola (GT73)	Monsanto	Spontaneous canola plants in ruderal habitats in the United States, including intervarietal hybrids with multiple transgenic events	Glyphosate resistance via 5-enolpyruvylshikimate-3-phosphate synthase and glyphosate oxidoreductase	Yes/Yes	Schafer et al. (2010)
2010	*Brassica napus* LibertyLink® canola (insufficient information to assign)	Bayer CropScience	Spontaneous canola plants in ruderal habitats in the United States, including intervarietal hybrids with multiple transgenic events	Glufosinate resistance via phosphinothricin N-acetyltransferase	Yes/Yes	Schafer et al. (2010)

[a]Center for Environmental Risk Assessment (2010)

canola in both Canada and Japan, different transgenic lineages have naturally crossed, resulting in some descendants with naturally "stacked", multiple transgenic deregulated events that belong to different companies (see citations in Table 7.1).

The entries in Table 7.1 span more than a decade. They involve plants created by both private and public entities. The handful of species involved range from annual agronomic crops to horticultural fruit trees to turfgrass. Herbicide tolerant *Brassica napus* canola is the species with the most entries. Its prevalence is likely due both to the facile dispersal biology of its seeds and pollen as well as ease with which herbicide tolerance can be assayed.

Equally interesting is what is NOT in the Table. Despite the fact that soybean and cotton are two of the world's four most important transgenic crops (James 2009), I could not find any reports that would justify inclusion in the Table. The only explanations that I can offer is that both of these crops are mostly self-pollinated, minimizing opportunities for unintended intervarietal crossing and neither founds feral populations, minimizing the opportunities for transgenes ending up in free-living populations. Other explanations may be equally plausible.

Contrary to initial concerns, crop transgenes have rarely moved into wild populations. Only two entries in Table 7.1 detail movement of transgenes into the wild: a deregulated event for herbicide tolerance from oilseed rape into populations of wild birdrape at two sites in Quebec, Canada (Warwick et al. 2003, 2008) and a *regulated* event for herbicide tolerance from a creeping bentgrass cultivar into wild populations of the same species in Oregon, USA (Reichman et al. 2006; Reichman and Watrud 2007; Zapiola et al. 2008). Research teams have been monitoring the frequency of both transgenes over time in natural populations. Warwick et al. (2008) found a significant decline in hybrid and hybrid-derived plants over a 6-year period, but the herbicide resistant trait associated with transgene was still present in those plants. In the case of herbicide resistant bentgrass, after 4 years, in wild populations the frequency of the phenotype associated with the transgene had increased to 62% (Zapiola et al. 2008). I am not aware of any other which transgenic crops have spontaneously mated with wild populations. But so far the two cases of transgene escape into the wild are more of academic interest than of any sort of environmental problem.

Escaped feral herbicide-resistant canola in Canada poses a different story. An initial biosafety review in Canada did not anticipate that transgenic canola volunteers would come feral because, at that time, canola had not been a problem plant (Canadian Food Inspection Agency 1994). As late as 2005, the predominant view was that 'In western Canada, herbicide-resistant volunteer *B. napus* is not at risk of becoming feral' (Hall et al. 2005). By 2010, it had become clear that transgenic volunteers had become "an increasing management problem in cultivated fields" and that populations in ruderal habitats near cultivated areas were persistent and evolving (Knispel and McLachlan 2010). In particular, volunteers descended from different canola varieties resistant to different herbicides have naturally hybridized to create multiple-herbicide resistant plants, not only in Canada, but in Japan as well (see Table 7.1 for citations). While hardly "superweeds", such plants create headaches for farmers who must use alternate, less desirable, herbicides to control them (Beckie et al. 2004).

Except, perhaps, the canola problem just discussed, the primary problems caused by transgenes out of place are not environmental ones. In many cases, unintended transgene release has simply been a matter of embarrassment and cleanup for the institutions involved. But the sheer number and diversity of incidents has created anxiety and contention about how to attain the coexistence of transgenic crops and those grown for transgene free markets (e.g., Altieri 2005; Ramessar et al. 2010). Finally, the growing number of incidents demonstrating that crop transgenes are more difficult to contain that previously anticipated has lead to the question of whether crops intended to produce pharmaceutical and other industrial compounds should be allowed to be grown outside (Nature Biotechnology 2002; Ellstrand 2003b; National Research Council 2004).

The question of crop transgenes out-of-place creates new questions for the evolution of regulatory policy. At one time, a small number of countries were, more or less, simultaneously deregulating the same, slowly growing list of new transgenic events. But now more countries are involved and the diversity of new transgenic products is growing. Lack of synchronization is creating regulatory asynchrony. In anticipation of one country's deregulated products ending up as 'contamination' in other countries where they have not yet been deregulated, policy makers are suggesting ways to avoid potential international gridlock of food and feed (Stein and Rodriguez-Cerezo 2010; Wager and McHughen 2010). For example, USDA-APHIS-BRS has proposed new regulatory guidelines for a codified policy to update their approach on dealing with the occurrence of very low levels of material that have not been fully deregulated within the United States (Jones 2009), what is now called 'low level presence' or 'LLP' (note: USDA-BRS-APHIS no longer uses the phrase "adventitious presence"). LLP could apply to a transgenic variety that has been deregulated elsewhere, but not the United States, or it could refer to a crop that has not been deregulated anywhere.

The issue of how to tolerate LLP is not straightforward. In some cases, unapproved events may be rather benign: quite similar to events already approved in the recipient country. Or the events may require questioning: different from anything yet deregulated in the world. The United States already employs a case-by-case evaluation of LLP (USDA 2007b) and as noted above is working to refine it. A "one size fits all" policy on LLP is unlikely to be embraced by any country.

Furthermore, regulatory policy should treat the processed products of transgenic crops differently from living organisms, such as grain, that, if planted, are capable of multiplying their genes. One percent LLP of rapeseed oil produced by an event as yet unapproved in a recipient country that is mixed with rapeseed oil from an approved event is an altogether different issue from 1% LLP of unapproved rape seed mixed with approved rape seed that may spill off a truck onto a suitable roadside habitat. The immigration of an advantageous allele at 1% frequency is evolutionarily significant, especially if the immigrant allele is constantly supplemented by repeated seed spillage (Ellstrand 2003a). Let's say that there may be some reason why the recipient country may not want a particular allele present in free-living populations. The persistence of feral canola populations in Japan, Canada, and the United States (see Table 7.1) presents an example that suggests that LLP tolerances

for living organisms should involve a case-by-case consideration; just as full deregulation involves a case-by-case evaluation in the United States, Canada, and elsewhere. This conundrum is one more example of the tension between free trade versus regulatory sovereignty (sometimes directed by science, sometimes guided by other factors) that continues as the twenty-first century unfolds.

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References

Altieri M (2005) The myth of coexistence: why transgenic crops are not compatible with agroecologically based systems of production. Bull Sci Technol Soc 25:361–371

Aono MS, Wakiyama M, Nagatsu N, Nakajima M, Tamaoki AK, Saji H (2006) Detection of feral transgenic oilseed rape with multiple-herbicide resistance in Japan. Environ Biosafety Res 4:217–222

Beckie HJ, Warwick SI, Nair H, Séguin-Swartz G (2003) Gene flow in commercial fields of herbicide-resistant canola (*Brassica napus*). Ecol Appl 13:1276–1294

Beckie HJ, Séguin-Swartz G, Nair H, Warwick SI, Johnson E (2004) Multiple herbicide-resistant canola can be controlled by alternate herbicides. Weed Sci 52:152–157

Bucchini L, Goldman LR (2002) Starlink corn: a risk analysis. Environ Health Perspect 110:5–12

Canadian Food Inspection Agency (1994) The biology of *Brassica napus* L. (Canola/Rapeseed). Biology document BIO1994-09. Plant Biosafety Office, Plant Products Directorate, Canadian Food Inspection Agency, Ottawa

Center for Environmental Risk Assessment (2010) GM crop database. http://cera-gmc.org/index.php?action=gm_crop_database. Accessed 23 July 2010

Cohen JL, Paarlberg R (2004) Unlocking biotechnology in developing countries: a report from the field. World Dev 32:1563–1577

Colwell RE, Norse EA, Pimentel D, Sharples FE, Simberloff D (1985) Genetic engineering in agriculture. Science 229:111–112

Council for Agricultural Science and Technology (2007) Implications of gene flow in the scale-up and commercial use of biotechnology-derived crops: economic and policy considerations. Issue paper 37. Council for Agricultural Science and Technology, Ames

Demeke T, Perry DJ, Scowcroft WR (2006) Adventitious presence of GMOs: scientific overview for Canadian grains. Can J Plant Sci 86:1–23

Dyer GA, Serratos-Hernández JA, Perales HR, Gepts P, Piñeyro-Nelson A, Chávez A, Salinas-Arreortua N, Yúnez-Naude A, Taylor JE, Alvarez-Buylla ER (2009) Dispersal of transgenes through maize seed systems in Mexico. PLoS One 4:e5734. doi:10.1371/journal.pone.0005734

Ellstrand NC (2003a) Dangerous liaisons? When cultivated plants mate with their wild relatives. Johns Hopkins University Press, Baltimore

Ellstrand NC (2003b) Going to 'great lengths' to prevent the escape of genes that produce specialty chemicals. Plant Physiol 132:1770–1774

Flax Council of Canada (2010) Flax council of Canada announces industry stewardship program for farm saved seed. Flax Council of Canada news release, 12 Mar 2010

Friesen LF, Nelson AG, Van Acker RC (2003) Evidence of contamination of pedigreed canola (*Brassica napus*) in western Canada with genetically engineered herbicide resistance traits. Agron J 95:1342–1347

Goodman RM, Newell N (1985) Genetic engineering of plants for herbicide resistance: status and prospects. In: Halvorson HO, Pramer D, Rogul M (eds) Engineered organisms the environment: scientific issues. American Society for Microbiology, Washington, DC, pp 47–53

Greenpeace International (2007) GM contamination register report 2007. Greenpeace International, Amsterdam

Hall L, Topinka K, Huffman J, Davis L, Allen A (2000) Pollen flow between herbicide-resistant *Brassica napus* is the cause of multiple-resistant *B. napus* volunteers. Weed Sci 48:688–694

Hall L, Rahman MH, Gulden RH, Allen AG (2005) Volunteer oilseed rape – will herbicide-resistance traits ferality? In: Gressel J (ed) Crop ferality and volunteerism. CRC Press, Boca Raton, pp 59–79

James C (2009) Global status of commercialized biotech/GM crops: 2009. ISAAA brief 41. ISAAA, Ithaca

Jones P (2009) APHIS restructures regulation of GE organisms. ISB news report special issue on US and EU Agbiotech regulation, pp 1–4

Kelly AF, George RAT (1998) Encyclopaedia of seed production of world crops. Wiley, Chicester

Kershen DL, McHughen A (2005) Adventitious presence. CAST Commentary. QTA2005-1. Council for Agricultural Science and Technology, Ames

Knispel AL, McLachlan SM (2010) Landscape distribution and persistence of genetically modified oilseed rape (*Brassica napus*) in Manitoba, Canada. Environ Sci Pollut Res 17:13–25

Knispel AL, McLachlan SM, Van Acker RC, Friesen LF (2008) Gene flow and multiple herbicide resistance in escaped canola populations. Weed Sci 56:72–80

Ledford H (2007) Out of bounds. Nature 445:132–133

Manshardt RM, Mello CL, Lum SD, Ta L (2007) Tracking papaya pollen movement with the GUS transgene marker. In: Proceedings of the first international symposium on papaya. Acta Hort 740:183–187

McHughen A (2006) Genetic engineering and testing methodologies. In: Agricultural biotechnology in California series, ANR publication 8190. University of California Division of Agricultural and Natural Resources, Oakland

National Research Council (1989) Field testing genetically modified organisms: framework for decisions. National Academy Press, Washington, DC

National Research Council (2004) Biological confinement of genetically engineered organisms. National Academy Press, Washington, DC

Nature Biotechnology (2002) Going with the flow. Nat Biotechnol 20:527

Nishizawa T, Nakajima N, Aono M, Tamaoki M, Kubo A, Saji H (2009) Monitoring the occurrence of genetically modified oilseed rape along a Japanese roadside. Environ Biosafety Res 8:33–44

Organization for Economic Co-operation and Development (2010) OECD seed schemes “2010”. Organization for Economic Co-operation and Development, Paris

Ortiz Garcia S, Ezcurra E, Schoel B, Acevedo F, Soberon J, Snow AA (2005) Absence of detectable transgenes in local landraces of maize in Oaxaca. Proc Natl Acad Sci USA 102:12338–12343

Piñeyro-Nelson A, Van Heerewaarden J, Perales HR, Serratos-Hernández JA, Rangel A, Hufford MB, Gepts P, Garay-Arroyo A, Rivera-Bustamante R, Alvarez-Buylla ER (2009) Transgenes in Mexican maize: molecular evidence and methodological considerations for GMO detection in landrace populations. Mol Ecol 18:750–761

Quist D, Chapela I (2001) Transgenic DNA introgressed into maize landraces in Oaxaca. Nature 414:541–543

Ramessar K, Capell T, Twyman RM, Christou P (2010) Going to ridiculous lengths: lengths—European coexistence regulations for GM crops. Nat Biotechnol 28:134–136

Reichman JR, Watrud LS (2007) Identification of escaped transgenic creeping bentgrass in Oregon. ISB News Repor Apr 1–4

Reichman JR, Watrud LS, Lee EH, Burdick CA, Bollman MA, Storm MA, King GA, Mallory-Smith C (2006) Establishment of transgenic herbicide-resistant creeping bentgrass (*Agrostis stolonifera* L.) in nonagronomic habitats. Mol Ecol 15:4243–4255

Saji H, Nakajima N, Aono M, Tamaoki M, Kubo A, Wakiyama S, Hatse Y, Nagatsu M (2005) Monitoring the escape of transgenic oilseed rape around Japanese ports and roadsides. Environ Biosafety Res 4:217–222

Schafer MG, Ross AX, Londo JP, Burdick CA, Lee EH, Travers SE, Van de Water PK, Sagers CL (2010) Evidence for the establishment and persistence of genetically modified canola populations in the U.S. In: Proceedings of the 95th meeting of the ecological society of America. http://eco.confex.com/eco/2010/techprogram/P27199.HTM

Serratos-Hernández JA, Gómez-Olivares JL, Salinas-Arreortua N, Buendía-Rodríguez E, Islas-Gutiérrez F, de Ita A (2007) Transgenic proteins in maize in the soil conservation area of federal district, Mexico. Front Ecol Environ 5:247–252

Stein AJ, Rodriguez-Cerezo E (2010) Low-level presence of new GM crops: an issue on the rise for countries where they lack approval. AgBioForum 13:173–182

Strayer D (2002) Identity-preserved systems: a reference handbook. CRC Press, Boca Raton

University of California Davis (2003) Tomato seed from seed bank found to be genetically modified. UC Davis news and information, 18 Dec 2003

USDA (2007a) Report of LibertyLink rice incidents. http://www.aphis.usda.gov/newsroom/content/2007/10/content/printable/RiceReport10-2007.pdf

USDA (2007b) APHIS policy on responding to the low-level presence of regulated genetically engineered plant materials. Federal Register, 72 FR 14649

USDA (2008) Questions and answers: genetically engineered corn "event 32". USDA APHIS Biotechnology Regulatory Services Fact Sheet

Wager R, McHughen A (2010) Zero sense in European approach to GM. EMBO Rep 11:258–262

Warwick SI, Simard M-J, Légère A, Beckie HJ, Braun L, Zhu B, Mason P, Séguin-Swartz G (2003) Hybridization between transgenic *Brassica napus* L. and its wild relatives: *Brassica rapa* L., *Raphanus raphanistrum* L., *Sinapis arvensis* L. and *Erucastrum gallicum* (Willd.) O. E. Schulz. Theor Appl Genet 107:528–529

Warwick SI, Légère A, Simard M-J, James TJ (2008) Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy *Brassica rapa* population. Mol Ecol 17:1387–1395

Zapiola ML, Campbell CK, Butler MD, Mallory-Smith CA (2008) Escape and establishment of transgenic glyphosate-resistant creeping bentgrass (*Agrostis stolonifera*) in Oregon, USA: a 4-year study. J Appl Ecol 45:486–494

Chapter 8
The Regulation of Organisms Used in Agriculture Under the *Canadian Environmental Protection Act, 1999*

Heather Darch and Arash Shahsavarani

Abstract The *Canadian Environmental Protection Act, 1999* is an Act respecting pollution prevention and the protection of the environment and human health, in order to contribute to sustainable development. Under this Act, living organisms, including micro-organisms and higher organisms, that are subject to the *New Substances Notification Regulations (Organisms)*, are required to be assessed for environmental and human health risks prior to their import into, or manufacture in Canada. In this Chapter we provide a summary of the notification and risk assessment process, and provide some examples of organisms used in agriculture that may be subject to the Act and Regulations. This Chapter will also highlight certain exemptions from the Act and Regulations which are aimed at minimizing regulatory duplication and to facilitate research and development activities in biotechnology, provided adequate safety measures are in place.

Keywords Canadian Environmental Protection Act, CEPA • Micro-organisms • Novel traits • Regulations • Risk assessment • Toxic

8.1 Part 6 of the *Canadian Environmental Protection Act, 1999*

The purpose of the *Canadian Environmental Protection Act, 1999* (CEPA 1999, or the Act) is to prevent pollution and to protect the environment and human health, in order to contribute to sustainable development. Part 6 of the Act (entitled *Animate*

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Products of Biotechnology) gives the Minister of the Environment the authority to require the notification and assessment of living organisms that are new to Canada prior to their import into or manufacture in Canada (subject to CEPA 1999 risk assessment triggers). This pre-import, pre-manufacture assessment gives the Minister the ability to intervene in the earliest stages of introduction into Canada to proactively prevent environmental and human health impacts in Canada that could result from exposure to these new organisms. One unique characteristic of CEPA 1999 is that it legally binds the Minister of the Environment and the Minister of Health with the responsibility to conduct a risk assessment to determine if a notified organism is toxic[1] as defined under section 64 of the Act.

A living organism is defined in Part 6 of the Act as a substance that is an animate product of biotechnology. Living organisms can include naturally occurring organisms used for a scientific purpose (such as bioremediation), or those that have been intentionally manipulated/created through science (such as genetically modified organisms). Both micro-organisms and higher organisms are captured under Part 6 of the Act.

Only "new" organisms that are proposed for import into or manufacture in Canada are subject to the requirements of Part 6 of the Act. The sole basis for determining whether a living organism is "new" is the Domestic Substances List (DSL). The DSL identifies organisms considered to exist in Canadian commerce. Organisms on this list do not require notification under CEPA 1999 prior to import into or manufacture in Canada.[2]

In Canada, the import, manufacture or use of a living organism and/or product containing a living organism may trigger regulatory oversight and/or risk assessment by a number of different statutes, depending on the intended use and/or product claims being made. To avoid regulatory duplication, Schedule 4 of CEPA 1999 identifies other Acts and Regulations that require an environmental and human health risk assessment equivalent to that conducted under CEPA 1999. Living organisms that are being used for a purpose that falls under the scope of one of the Acts or Regulations listed in Schedule 4, do not require notification under CEPA 1999 prior to import or manufacture for that specific use. For example, living organisms imported or manufactured for use as a pest control product, feed, fertilizer or veterinary biologic are subject to the requirements of the *Pest Control Products Act*, *Feeds Act*, *Fertilizers Act*, or *Health of Animals Act*, respectively, and therefore are not

[1] Toxic is defined in Part 5 of CEPA 1999. An organism is considered to be toxic if it is entering or may enter the environment in a quantity or concentration or under conditions that (a) have or may have an immediate or long-term harmful effect on the environment or its biological diversity, (b) constitute or may constitute a danger to the environment on which life depends; or (c) constitute or may constitute a danger in Canada to human life or health.

[2] Although not listed on the DSL, common domesticated animals and higher organisms that are indigenous to Canada are also considered to be existing and do not require notification and assessment prior to import or manufacture. Where an organism is listed on the DSL with a "Significant New Activity" (SNAc) flag, re-notification is required if the organism is proposed to be used for a Significant New Activity as outlined in a SNAc (see Sect. 8.6 for further explanation).

subject to notification requirements under CEPA 1999. Likewise, the *Seeds Act* and *Seeds Regulations* are also listed in Schedule 4 of CEPA 1999. Therefore, only plants with novel traits (PNT) falling outside of the scope of the *Seeds Act* and *Seeds Regulations*, such as PNT imported for processing only and not for planting, may be subject to CEPA 1999. Requirements for PNTs under the *Seeds Act* and *Seeds Regulations* are discussed further in Chap. 9.

Section 114 of CEPA 1999 gives the Minister of the Environment and the Minister of Health the authority to recommend regulations related to organisms or groups of organisms. The *New Substances Notification Regulations (Organisms)* [NSNR (Organisms) or the Regulations] were created under this authority, to implement Part 6.

8.2 The New Substances Notification Regulations (Organisms)

The NSNR (Organisms) were published in *Canada Gazette, Part II* on September 21, 2005 and came into force on October 31 of that same year. Prior to that date, living organisms were regulated since 1997 in a similar manner under the *New Substances Notification Regulations* that contained provisions for organisms, chemicals and polymers. The NSNR (Organisms) implements Part 6 of CEPA 1999 and prescribes exemption criteria, information requirements, and timelines for notification and assessment of new living organisms.

8.2.1 Exemptions

The NSNR (Organisms) identifies a number of organisms that are exempt from the Regulation. Subsection 2(1) of the Regulation exempts organisms that are manufactured or imported for a use that is regulated under any Act or Regulation listed in Schedule 4 of CEPA 1999, as described in section 8.1 above. Subsection 2(2) of the Regulation exempts organisms that are in transit (loaded on a carrier outside of Canada and moved through Canada to a location outside of Canada, whether or not there is a change of carrier during transit).

Subsections 2(3) and 2(4) exempt micro-organisms and higher organisms, respectively, that meet the definition of a research and development (R&D) organism,[3] are kept in containment and in the case of micro-organisms, meet certain

[3] Research and development organism is defined in subsection 1(1) of the NSNR (Organisms) as an organism that is undergoing systematic investigation or research, by means of experimentation or analysis other than test marketing, whose primary objective is any of the following: (a) to create or improve a product or process; (b) to determine the technical viability or performance characteristics of a product or process; or (c) to evaluate the organism prior to its commercialization, by pilot plant trials, production trials, including scale-up, or customer plant trials so that technical specifications can be modified in response to the performance requirements of potential customers.

threshold volumes. Appropriate containment can be achieved through national or international standards.

For micro-organisms, containment guidelines such as the Public Health Agency of Canada's *Laboratory Biosafety Guidelines* or Appendix K of the NIH Guidelines[4] are recognized as acceptable for adequate containment when applied correctly based on the risk level[5] of the micro-organism. Volume thresholds are also in place and in order to be exempt from notification, the Regulations require that (a) import volumes into a contained facility be in a quantity of less than 50 mL or 50 g; (b) manufactured quantities of the micro-organism at any one time be less than 1,000 L; (c) where there is a requirement for containment level 2, manufacture and presence at any one time in a contained facility is less than 250 L; or (d) if the organism is a human pathogen and a level 3 or 4 containment is required, manufacture or presence of the micro-organism at any one time in a contained facility is less than 250 L and a permit or an approval in writing to transfer has been granted under the *Human Pathogen Importation Regulations*.

For higher organisms that are R&D organisms, the organisms must be manufactured in or imported to a facility from which there is no release into the environment of (a) the organism; (b) the genetic material of the organism; or (c) material from the organism involved in toxicity. In 2010, an Advisory Note was released by Environment Canada to clarify the requirements for exemption under subsection 2(4) in particular to elaborate the type of genetic and hazardous material that needs to be contained,[6] including gametes, unattenuated viral vectors, transposons with functional transposase genes, toxins etc.

8.2.2 Information Requirements and Timelines for Notification and Assessment

Information requirements for notifying a micro-organism are identified in one of the first four Schedules of the NSNR (Organisms), and are dependent on the intended use. Information requirements for organisms other than micro-organisms (i.e. higher organisms) are listed in Schedule 5 of the Regulations. Higher organisms are identified as those organisms that do not meet the regulatory definition of a micro-organism. For the purpose of the regulations, micro-organisms include, but are not limited to, organisms classified as Bacteria, Archaea, Protista (including protozoa and algae), fungi (including yeasts), and viruses, virus-like or sub-viral particles.

Upon receipt of a notification that contains all of the prescribed information, evaluators from Environment Canada and Health Canada conduct a joint risk assessment to

[4] "NIH Guidelines" means the *Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines) June 1994.* Published in the Federal Register by the U.S. Department of Health and Human Services, 59 FR 34472 (July 5, 1994), as amended from time to time.

[5] As established by the Public Health Agency of Canada.

[6] The Advisory Note may be found at the following website: http://www.ec.gc.ca/subsnouvelles-newsubs/default.asp?lang=En&n=FB69B50A-1

Table 8.1 Schedules of information requirements for micro-organisms notified under the NSNR (Organisms)

Schedule number	Use	Timeframe for notification
1	Micro-organisms for release into the environment including release into specific ecozones or with confinement procedures	120 days
2	Micro-organisms not for introduction outside a contained facility or are for export only	30 days
3	Micro-organisms for introduction in an experimental field study	90 days
4	Micro-organisms manufactured at a site from which they were isolated for introduction into the same site	30 days

determine whether or not the organism is toxic or capable of becoming toxic.[1] This risk assessment must be completed within time-lines prescribed in the NSNR (Organisms) and range from 30 days for a contained activity with a micro-organism to 120 days for an activity resulting in full release of the micro-organism in the Canadian environment (Table 8.1). For an activity involving an organism other than a micro-organism (i.e. a higher organism), the assessment timeline is 120 days. Both direct environmental effects and indirect[7] human health effects are taken into consideration when arriving at the final assessment conclusion. Import or manufacture of the organism may only begin once the assessment period has expired and any necessary risk management measures for organisms found to be toxic, are in place.

It should be noted that importers or manufacturers may have other requirements prior to import, manufacture or use of an organism in Canada. For example, import permits for plant, animal or human pathogens would still be required from appropriate authorities; importers or manufacturers of a living organism may also have requirements under other Acts not listed in Schedule 4 of CEPA 1999, such as the *Food and Drugs Act* for an assessment of direct human health effects.

8.3 Micro-organisms Used in Agriculture, Subsection 2(3) and Section 3 of the NSNR (Organisms)

Although the scope of micro-organisms captured under the NSNR (Organisms) is greater than just those used in agriculture, this section will focus on those micro-organisms that are used for an agricultural purpose. As mentioned in Sect. 8.1,

[7] Indirect human health effects refer to human health effects resulting from an environmental release of the living organism.

micro-organisms that are used in a pest control product, or that meet the definition of a feed or fertilizer, are not captured under the NSNR (Organisms) as these are subject to an equivalent environmental, health and safety risk assessment under other Canadian legislations. Nonetheless, there are some micro-organisms that may have applications in the agricultural industry that will require notification under the NSNR (Organisms) prior to import or manufacture. For example, micro-organisms used to catalyze or hasten the decomposition in a compost pile or those used as odour control agents in compost piles and manure pits may be subject to the NSNR (Organisms) and risk assessment under CEPA 1999.

Micro-organisms requiring notification are subject to subsection 3(1) of the NSNR (Organisms). These are micro-organisms that are not on the DSL, and that do not meet the research and development exemption criteria prior to import or manufacture. Depending on the proposed use, one of four different Schedules in the Regulations may apply to the micro-organism and all information requirements listed in that Schedule must be provided (Table 8.1). For example, a micro-organism for use as a compost accelerant and with an intention to be sold to farmers throughout Canada would require a notification under Schedule 1. A micro-organism used in a closed system ethanol fermentation plant using agricultural residues as biomass would be notifiable under Schedule 2. A micro-organism used to test disease resistant crops in an open field trial would be notifiable under Schedule 3 and a micro-organism isolated from a field and re-introduced to the same field to degrade pesticide residues would be notifiable under Schedule 4. All of the information specified under the appropriate Schedules would need to be provided at least 120, 30, 90 or 30 days, respectively, prior to the date import or manufacture was planned to commence. Effectively, import or manufacture could not actually begin, until the assessment period (120, 30, 90, or 30 days) has expired.

8.4 Higher Organisms Used in Agriculture Subsection 2(4) and Section 4 of the NSNR (O)

Unlike the micro-organism portion of the NSNR (Organisms) that identifies various Schedules of information requirements based on the use of the micro-organism, all importers and manufacturers of new higher organisms are required to submit a notification under Schedule 5 of the Regulations unless exemption criteria apply (see Sects. 8.1 and 8.2.1 above). Schedule 5 is a generic schedule of requirements meant to capture the import or manufacture of any higher organism for any purpose. Import or manufacture could not actually begin until a completed notification package has been submitted to Environment Canada and the assessment period (120 days) expires.

Examples of higher organisms that would require notification under the NSNR (Organisms) that are used in agriculture would include, but are not limited to, genetically modified fish and livestock that are imported live, or are manufactured/

produced in Canada. Examples of such organisms would include the Enviropig™ that was assessed in 2009,[8] genetically modified seed for industrial processing, and genetically modified fish.

8.5 The Risk Assessment Process

The risk assessment process, outcomes and risk management measures described below are applicable to all organisms subject to the NSNR (Organisms) whether they are micro-organisms or higher organisms. The initial source of information used in the risk assessment is that which is provided by a proponent through the notification package, but evaluators also use in-house information and any additional information available in the public domain. This information helps to assist the evaluators in determining both potential hazards associated with the organism and potential pathways of exposure to them, in order to estimate risk and the likelihood that an organism is toxic or capable of becoming toxic as defined under section 64 of the Act.[1] Therefore section 64 effectively provides the endpoints to be addressed through a risk assessment. If necessary, evaluation teams may contact external experts (both in Canada and abroad) to further inform the risk assessment without divulging any confidential business information. These consultations are generally driven by specific technical or scientific questions posed by the evaluation teams and are only used when in-house expertise can not adequately address uncertainties.

The information elements listed in each schedule provide the minimum information that must be provided by the proponent so that evaluation staff may begin the risk assessment. In broad terms, the information required to be provided in a notification package includes (a) information in respect of the identification and characteristics of the organism (including any modifications), (b) the manufacture and import details of the organism, (c) the introduction of the organism into the environment (which includes any measures, such as containment measures in place to prevent introduction into the environment), (d) the environmental fate of the organism, (e) the ecological effects of the organism and (f) the human health effects of the organism.

The evaluation and risk assessment is a joint responsibility between two Ministers; evaluators from Environment Canada conduct an assessment for environmental effects including biodiversity, while evaluators from Health Canada conduct an assessment for indirect human health effects (that is, exposure resulting from the environmental release of the organism). As mentioned earlier, direct human health effects may be assessed under other Legislation, such as the *Food and Drugs Act* as appropriate. The ability to import or manufacture under CEPA 1999 does not exempt the notifier from any other laws or regulations that are in force in Canada and that may apply to the organism or activities involving the organism.

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[8] Link to SNAc Notice for Enviropig™ http://canadagazette.gc.ca/rp-pr/p1/2010/2010-02-20/html/notice-avis-eng.html#d103. Accessed 5 Sept 2011.

8.6 Possible Risk Assessments Outcomes and Risk Management Measures

There are three possible outcomes as a result of a risk assessment (Fig. 8.1). First, if there is no suspicion that the new organism is toxic or capable of becoming toxic, the notifier may proceed with import or manufacture after the assessment period has expired.

A second possible outcome is that one or both Ministers[9] suspect that the organism may become toxic should it be used for an activity that is significantly different from that which has been proposed and assessed, in other words for a Significant New Activity (SNAc). In such cases, the government has the authority to require re-notification and assessment prior to import or manufacture for a significant new use. In addition to identifying the organism to which the SNAc applies, the Notice typically also includes a description of what constitutes a significant new activity with respect to the assessed organism. The Notice will also generally include the new information requirements for the Significant New Activity Notification (SNAN) and the timeline within which the assessment would take place.

The third possible outcome of a risk assessment is a suspicion by either Minster that the organism is or may become toxic under section 64 of the Act. In response to this risk assessment outcome, control measures may be applied to minimize any risk to the environment, biological diversity or human health. These may include permission to manufacture or import subject to any conditions that the Ministers may specify; prohibition from manufacture or import requiring the development of specific regulations for the organism within 2 years, or prohibition of manufacture or import until supplementary information or test results have been submitted and assessed.

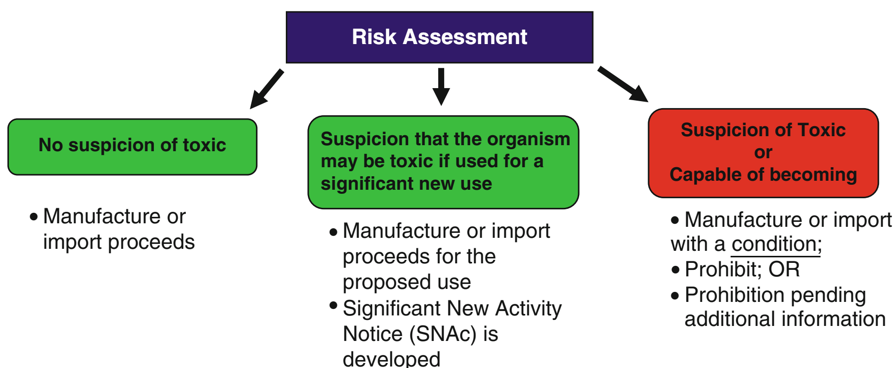

Fig. 8.1 Possible risk assessment outcomes under CEPA 1999

[9] Minister of Environment and Minister of Health.

Organisms that have been assessed under a full Schedule 1 or 5, without any information requirements waived on the basis of limited exposure may be eligible for addition to the DSL unless conditions are in place on the use of the organism. Once on the DSL, notification is no longer required in advance of import or manufacture unless proposed for a significant new activity as specified (or flagged) on the DSL.

8.7 Conclusion

Although organisms have been used for centuries in the food industry, the growth in biotechnology and move towards a bio-based economy will lead to new applications of, and exposures to, a variety of new organisms. The *Canadian Environmental Protection Act, 1999* and the *New Substances Notification Regulations (Organisms)* are critical pieces of Legislation that allow the Canadian Government to protect the environment and the health of Canadians, by allowing risk identification, assessment and intervention prior to or during the earliest stages of an organisms' introduction to Canada.

Chapter 9
Regulating the Environmental Release of Plants with Novel Traits in Canada

Krista Thomas and Stephen Yarrow

Abstract This chapter provides an overview of the regulation of the environmental release of plants with novel traits (PNTs) in Canada. The development of the Canadian biotechnology regulatory framework, the specifics of confined and unconfined release of PNTs, including the application of Canada's unique regulatory trigger and approach to stacked products, and future challenges are discussed.

Keywords Regulation • Biotechnology • Plants with Novel Traits (PNTs) • Canada • Environmental Release

9.1 Introduction

It has been over two decades since the first research field trials of plants developed using recombinant DNA techniques occurred in Canada. Since this time, biotechnology-derived plants, particularly canola, corn and soybean have been widely adopted by Canadian farmers. Canada is now the world's fifth largest producer of biotechnology-derived crops, with 8.2 million ha planted in 2009 (James 2009).

As with the introduction of any new technology, the adoption of agricultural biotechnology has been accompanied by an active public dialogue about the real and perceived potential for adverse effects, including impacts on the environment.

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As a result, the regulatory framework for biotechnology that has evolved in Canada strives to balance the benefits offered by these products for Canadians, with a science-based approach to managing their potential environmental risks.

9.2 The Evolution of Canada's Biotechnology Regulatory Framework

In the late 1980s, many researchers working on plant biotechnology in laboratories across Canada were already familiar with the function of the regulatory arm of Agriculture and Agri-Food Canada (AAFC), the Food Production and Inspection Branch. At that time, AAFC was responsible for regulating the registration of new varieties, based on an assessment of merit. It had done so since 1923 under the authority of the *Seeds Act*, which governs the testing, inspection, quality and sale of seeds in Canada. The delivery of the these existing regulatory programs involved activities such as overseeing field testing of new plants under controlled conditions to minimize cross pollination and to maintain high levels of seed purity during the tests.

AAFC's regulatory experience also included the application of risk mitigation measures in order to prevent the entry of potentially destructive pest organisms into the country. Under such legislative antecedents as the *Canadian Destructive Insect and Pest Act* of 1910, federal officials conducted risk assessments to determine the potential adverse impact on the native flora and agricultural base before an import was approved.

Intuitively, researchers from private companies, universities and public institutions turned to AAFC seeking regulatory direction regarding the new plants they were producing using the techniques of biotechnology.

In the course of assuming this new regulatory responsibility, AAFC conducted a number of multi-stakeholder consultations to seek advice on the regulatory scope and approach that should be used to govern activities such as importation, field research and cultivation of biotechnology-derived plants. During this period, environmental interest groups such as the Canadian Environmental Network and Pollution Probe also began to approach AAFC with their concerns.

In 1988 AAFC organized a consultation in conjunction with the Canadian Agricultural Research Council (CARC), a non-profit organization of researchers from industry, academia, federal and provincial governments. The workshop participants made a seminal recommendation that has become the basis for the Canadian regulatory approach for products of biotechnology. They recommended that regulations for these new plant should be focused on those "which possess characteristics, or traits sufficiently different from the same, or similar species, as to require an assessment of risk." This led to the recommendation that "the product, and not the process" be regulated, i.e., that it was the presence of a novel trait in a plant that potentially posed environmental risk, and not how the traits were specifically introduced. A comparison of the modified plant with unmodified counterparts to assess risk was a logical extension. These recommendations were consistent

with other international guidance developed at this time (OECD 1986; National Academy of Sciences 1989). Over time, this recommendation has led to the regulation in Canada of a few of the new plant varieties developed through mutagenesis breeding in much the same manner as varieties developed using recombinant DNA (rDNA) technology.

During the same period, the *Canadian Environmental Protection Act*, 1988 (CEPA 1988) was promulgated, adding additional regulatory requirements with respect to environmental protection, in a number of areas including biotechnology. However, as a consequence of the AAFC-CARC consultations, and AAFC's long experience with variety registration trials, applications to conduct Canada's first experimental plantings of "plants with novel traits" (PNTs) were regulated by AAFC. This continued until the creation of the Canadian Food Inspection Agency (CFIA) in 1997, at which time the responsibility passed to the Agency.

The development of CEPA 1988 created the need for Environment Canada and AAFC, in the early days of regulating research field trials of PNTs, to find an appropriate and pragmatic solution to avoid unnecessary duplication in regulations and responsibilities. In the late 1980s and early 1990s much debate ensued both within government and among stakeholders as to how the government regulation of field research on PNTs should continue. Stakeholders recognized that AAFC had the necessary expertise in agriculture and agronomy, ecology, and molecular biology to regulate these field trials. However, new regulations under the authority of the *Seeds Act* needed to be developed to specifically require the notification and environmental assessment of these new plants. Guiding the development of these new regulations would be emerging federal policy guidance on biotechnology.

The Government of Canada approved a federal regulatory Framework for Biotechnology in 1993 (Government of Canada 1993). The Framework called for the benefits of biotechnology products and processes to be realized in a way that would protect the environment, human health and safety, and resulted from an agreement among federal regulatory departments and agencies to the following six key principles:

- to maintain Canada's high standards for the protection of the health of workers, the general public and the environment
- to use existing legislation and regulatory institutions to clarify responsibilities and avoid duplication
- to continue to develop clear guidelines for evaluating products of biotechnology which are in harmony with national priorities and international standards
- to provide for a sound scientific basis on which to assess risk and evaluate products
- to ensure both the development and enforcement of Canadian biotechnology regulations that are open and include consultation
- to contribute to the prosperity and well-being of Canadians by fostering a favourable climate for investment, development, innovation and adoption of sustainable Canadian biotechnology products and processes

Along with addressing new plants, the Framework applied to all products that may be developed using biotechnology such as foods, livestock feeds, veterinary vaccines, pest control products and fertilizers.

Interestingly, the Framework called for reliance on existing legislation and institutions rather than the development of a "Gene Act" or the establishment of a "Biotechnology Agency." This meant that novel products such as PNTs would be regulated in a consistent manner, broadly speaking, to conventionally-derived products. This guidance was influential on the development of a new part of the *Seeds Regulations* (Part V) to govern the environmental release of PNTs. During the same period, other regulations such as those pertaining to the food and livestock feed safety of novel products were also being developed. Although they are beyond the scope of this chapter, see Chaps. 2 and 8 for related information.

Part V of the *Seeds Regulations*, published in 1996, prohibits the environmental release of a PNT in Canada unless written notice of the proposed release is provided to the Minister of Agriculture. The notice must be accompanied by prescribed information before authorization of the release may be granted.

AAFC and Environment Canada undertook extensive consultations and negotiations leading up to 1996, to produce the final version of these new regulations, which provide a mechanism to authorize both confined research field trials of PNTs, as well as unconfined environmental release, a regulatory step that product developers would need to complete (in addition to any other applicable regulatory processes, such as variety registration) prior to commercial cultivation of a PNT in Canada. In order to ensure consistency with CEPA 1988, the new seeds regulations incorporated the same definition of biotechnology and of toxicity. Specifically, Subsection 107(2) of the *Seeds Regulations* Part V states:

> *For the purposes of this Part, seed is toxic if it is entering or may enter the environment in a quantity or concentration or under conditions:*

(a) *having or that may have an immediate or long-term harmful effect on the environment;*
(b) *constituting or that may constitute a danger to the environment on which human life depends; or*
(c) *constituting or that may constitute a danger in Canada to human life or health*

A renewed CEPA was promulgated in 1999 (CEPA 1999) becoming the key authority used by the Government of Canada to ensure that all new substances (including organisms derived using biotechnology) are assessed for their potential to harm human health or the environment. However, recognizing that other Canadian legislation also provides for such an assessment process, CEPA includes a provision whereby substances or organisms regulated by other equivalent Acts are exempt from the new substance notification requirements of CEPA. This avoids regulatory duplication, while making sure that standards for protection of the environment and human health are met (Environment Canada 2001). By 2001, the *Seeds Act* and *Regulations* had been listed in Schedule 4 of CEPA 1999. This meant that the responsibility for regulating the environmental release of seed of all PNTs including food crops, trees, horticultural, and marine plants (any member of the plant kingdom) would rest with the CFIA under the authority of the *Seeds Act* and *Regulations*, and would be exempt from regulation by Environment Canada under CEPA 1999.

During this period, Canada's Biotechnology Regulatory Framework continued to evolve. The six principles of the Framework, first announced in 1993, were confirmed

and renewed in 1998, as part of a new Canadian Biotechnology Strategy (CBS) (Government of Canada 1998). This strategy also included a dialogue on a broader range of emerging issues relating to ethics, social policy and environmental safety, stimulated by the accelerating pace in biotechnology developments and adoption in Canada.

A component of the CBS was the creation of the Canadian Biotechnology Advisory Committee (CBAC), an expert committee to provide advice to the government on emerging issues, and to facilitate the incorporation of public input into the strategy. Augmenting the CBAC was the establishment of an "Expert Panel on the Future of Food Biotechnology" under the auspices of the Royal Society of Canada. Conferring with the Ministers of Agriculture, Health and the Environment, the Panel was asked to provide advice on a series of questions related to the safety of new food products being developed through the use of new genetic engineering technologies (The Royal Society of Canada 2001). The CFIA and other government departments responded to the recommendations of the Royal Society with an action plan (Government of Canada 2001) that was implemented between 2001 and 2005. A key commitment for the CFIA and other departments resulting from this advice was to increase the transparency of the regulatory process for products of biotechnology.

One such measure to improve regulatory transparency was implemented in 2004, when the CFIA and Health Canada began posting on the CFIA website "notices of submission" at the time that developers of PNTs, novel foods and novel livestock feeds first submit their products for regulatory review. These notices describe the product and summarize the type of data the product developers included in their submission data package. This information is made available by product developers on a voluntary basis, providing an opportunity for the public to provide input on scientific matters relevant to the safety assessment of each product, before regulatory decisions are made (CFIA 2004a).

In addition to the aforementioned processes to review and improve Canada's approach to regulating biotechnology, specific regulatory guidance documents and policies have been updated by regulators on a continuous basis, in accordance with new science and in response to practical experience gained following the adoption and large-scale cultivation of biotechnology-derived crops in Canada. For example, in recent years the CFIA has made clarifications to its policies related to the environmental release of stacked products, and the interpretation of Canada's unique regulatory trigger and definition of a PNT.

9.3 The Application of Novelty: Canada's Unique Regulatory Trigger for PNTs

Canada's use of the "novelty trigger," rather than a processed-based, rDNA trigger for environmental safety reviews of PNTs is a science-based approach that applies the same regulatory standard to all plant breeding techniques, leaving breeders free to use the most appropriate method to meet their objectives. Whether a plant breeder uses rDNA technology, mutagenesis or another plant breeding technique, the resulting

plant variety may or may not trigger an environmental safety assessment under Part V of the *Seeds Regulations*. However, some Canadian plant breeders have raised concerns about the impact of this approach on competitiveness and innovation, and its predictability. Each new crop variety developed by a plant breeder is genetically new and different in some way. In which cases does a new variety require notification and safety assessment by the CFIA prior to its environmental release?

Part V of the *Seeds Regulations* provides a definition of a novel trait in subsection 107(1), whereby a *"novel trait, in respect of a seed, means a characteristic of the seed that:*

(a) *has been intentionally selected, created, or introduced into a distinct population of cultivated seed of the same species through a specific genetic change, and*
(b) *based on valid scientific rationale, is not substantially equivalent, in terms of its specific use and safety both for the environment and for human health, to any characteristic of a distinct, stable population of cultivated seed of the same species in Canada, having regard to weediness potential, gene flow, plant pest potential, impact on non-target organisms, and impact on biodiversity.*"

The intention of Canada's use of novelty as a regulatory trigger is not to subject all new plant varieties to an unduly onerous regulatory process. Hundreds of new crop varieties or lines are introduced every year, without threat to the Canadian environment and without need of an environmental safety assessment conducted by federal regulators. The intent of focusing on plants that are "not substantially equivalent having regard to weediness potential, gene flow, plant pest potential, impact on non-target organisms and impact on biodiversity" is to capture only those plants with the greatest potential to have a negative impact on the environment within the definition of a PNT.

The CFIA clarified its regulatory trigger for PNTs through a series of consultations between 2005 and 2008, organized by the National Forum on Seed, a stakeholder group representing a spectrum of seed industry stakeholders (National Forum on Seed 2008). This work culminated with the CFIA's publication of a new guidance document in 2009 (CFIA 2009b). This document provides information particularly for use by conventional breeders to clarify when a new line or variety meets the definition of a PNT and triggers regulation under Part V of the *Seeds Regulations*. Although any new plant variety could meet the definition of a PNT, this directive provides increased predictability for breeders by identifying three breeding objectives that always require notification to the CFIA under the authority of Part V of the *Seeds Regulations*:

1. Any introduction of a new trait that significantly and negatively alters the sustainable management of the crop. For example:
   - Herbicide resistance/tolerance (where stewardship and/or volunteer management is important to delay the development of resistant/tolerant weeds)
   - Insect resistance (where stewardship is important to delay the development of resistant insect populations)

2. Any change to the plant which results in a novel production or accumulation of molecules that may have a harmful effect on living systems, e.g. those that are intended for pesticidal, pharmacological or industrial uses.
3. Any introduction of a new trait that may result in an increase in overall plant fitness or competitiveness in a crop for which Canada is a centre of diversity.

The document also provides guidance on determining if a new variety is a PNT in cases where the above three criteria do not apply. In addition, the process for engaging with the CFIA to confirm whether a plant is a PNT, as well as a recourse mechanism, are provided. In addition to this guidance, the CFIA and Health Canada encourage developers of PNTs, novel feeds and novel foods to make use of pre-submission consultations. These informal meetings with regulators provide a mechanism for plant breeders and technology developers to confirm the regulatory status of their products, as well as to discuss whether the research studies they have planned will adequately address the information required to complete the safety assessment process.

PNTs are defined by their characteristics, rather than their manner of production. This means that as techniques used by plant breeders continue to evolve, the regulatory trigger for PNTs will remain current, while process-based approaches used in other jurisdictions may be challenged or circumvented. However, this also means that not all plants developed through rDNA technology will necessarily meet the definition of a PNT. To date, the developers of all new plants produced in this manner have considered them to be regulated articles and have submitted them for CFIA review according to published guidance (including procedures for stacked products and retransformations). However, the future may well hold the possibility of plants developed through rDNA technology that are not PNTs. In this event, the CFIA will need to consider mechanisms to ensure that transparency and international accountability can continue to be adequately addressed. For example, Canada has committed to make information available on the Biosafety Clearinghouse, an international mechanism to exchange information about the movement of living modified organisms (LMOs), established under the Cartagena Protocol on Biosafety. To meet this commitment, knowledge of all of the LMOs that may be cultivated in Canada, regardless of whether they are PNTs, will be required.

9.4 The Regulation of the Environmental Release of PNTs into the Canadian Environment

Pursuant to the promulgation of *Part V* of the *Seeds Regulations* in 1996, all newly developed PNTs began to require notification and explicit authorization prior to their release into the environment. PNTs present in the Canadian environment prior to the coming into force of these regulations were exempted from the notification and other requirements of the regulation.

Environmental release is defined broadly in the regulation, as "any discharge or emission of seed into the environment or exposure of seed to the environment and includes the growing and field testing of plants (Government of Canada 1996)." As such, "release" may include a wide range of activities. However, the regulations further specify two categories of release:

- confined release into the environment, i.e., release under conditions intended to minimize the establishment and spread in the environment, of seed or of genetic material from plants derived from the seed, and the interaction of the seed or genetic material with the environment
- unconfined release into the environment, i.e., release on an unrestricted basis (though conditions may be applied to the authorization to manage risk).

These two release stages are closely connected because one purpose of a confined release is to allow the conduct of experiments designed to meet the information requirements necessary for applications for unconfined release.

9.4.1 Confined Release

Confined research field trials of PNTs provide applicants with the opportunity to evaluate the environmental safety of their PNTs in the field, under conditions of reproductive isolation. PNTs are typically grown in confined trials over a number of years, in multiple locations across Canada that are relevant to the cultivation of the crop. Product developers collect environmental and agronomic data during these trials to assist with event selection or to meet the information requirements for unconfined environmental release or other regulatory requirements. However, researchers wishing to do field studies on PNTs for purely academic purposes with no intention of producing a commercial variety may also make use of this program. The CFIA is not prescriptive about the type of research that may be conducted within a trial, however the CFIA does set strict terms and conditions that address reproductive isolation, site monitoring, disposal of material and post-harvest land use at the trial site.

Information about confined field trials that have been authorized in Canada, and their associated species-specific terms and conditions, is available on the CFIA web site and is updated on an annual basis (CFIA 2009d). In addition, detailed guidance on the operation of the confined research field trial program is available in *Regulatory Directive 2000–07: Conducting Confined Research Field Trials of Plants with Novel Traits in Canada*, available on the CFIA web site. At the time of writing, the most recent update to this document occurred in 2009 (CFIA 2009c). This directive provides instructions to applicants who are seeking either authorization or renewal of previously authorized confined field trials of PNTs.

The types of novel traits that have been involved in these research trials since 1988 include herbicide resistance, resistance to insect pests or plant pathogens, pollination control mechanisms, stress tolerances, changes in nutritional quality, and

production of high value substances, such as pharmaceuticals and industrial chemicals. The species involved include canola and other *Brassica* species, potatoes, corn, flax, soybeans, wheat, safflower, alfalfa, lentils, sugar beet, barley, broccoli, canary seed, grape vine, pea, perennial ryegrass, tobacco, tomato, white clover and several tree species. More than 8,000 confined trials of over 1,000 unique PNTs have been authorized in Canada since 1988. The average annual number has in recent years ranged between approximately 200 and 800 trials (CFIA 2009d).

The confined release assessment process is initiated when an application is submitted to the CFIA. Applications are usually submitted at least 30 days before the proposed planting dates to allow enough time for the CFIA to complete its review and make a decision regarding the proposed release. The CFIA has a policy of notifying designated provincial government contacts of trials proposed within their province. The CFIA will facilitate communication between the province and the trial applicant, in cases where additional information or discussion is required.

The overarching objective of the confined research field trial program is to minimize the spread and persistence of plant material at the trial site and to prevent the entry of a PNT into food or feed prior to its authorization for those uses. PNTs in a field trial must be grown in conditions of reproductive isolation from other plants of the same or related species, separated by specified isolation distances. These distances are based on knowledge arising from pollen flow studies that have been published in peer-reviewed literature or commissioned by the CFIA, as well as knowledge of traditional plant breeding practices and on recommendations established by the Canadian Seed Growers' Association for the production of certified seed. Practical considerations are also employed in setting isolation distances. Regardless of the biology of the plant, no confined research field trial may have less than a 10 m isolation distance. This requirement is based not on biology but on the practical logistics of planting and harvesting a trial, and the potential for seed dispersal through mechanical rather than biological means.

Other than physical isolation, different means of reproductive isolation may also be suitable for use. These may include termination of the trial prior to flowering, removal of flower buds prior to flower opening, the use of tents or mesh coverings to prevent pollen movement by insects, and guard rows of non-modified plants of the same species at a fixed depth and surrounding the experimental plants.

Each applicant authorized to conduct a trial is responsible for strictly adhering to a detailed set of terms and conditions that apply before, during and after harvest. Some of these conditions are species-specific. For example, isolation distances for canola confined trials are 200 m, whereas soybean is 10 m, reflecting differences in the outcrossing rates and pollen-flow distances of these two crop kinds. Other terms and conditions are common to all field trials. For example, typical field trial sites are limited in size to a maximum of 1 ha (ca. 2.4 acres), with no more than ten sites permitted per trial in any given year, although the CFIA will consider requests for exemptions of this requirement when the research objectives of the applicant require larger sites. The restrictions also greatly assist CFIA inspection staff in organizing their enforcement and compliance activities.

Examples of other general terms and conditions applied to confined research field trials include:

- applicants must ensure that all seed is transported in clearly identified, secure containers and are kept separate from other seed and/or plant material
- Seeding, transplanting and site maintenance machinery and equipment must be cleaned at the trial site to prevent dispersal of plant material.
- Global positioning system coordinates must be taken precisely at all corners of each trial site and must be submitted to the CFIA within 7 days after planting.
- A detailed trial log book must be kept. Records of the confined research field trial, including current season and post-harvest site monitoring, activities related to the trial site compliance, cleaning of machinery, transportation, disposition and storage of all surplus seed and harvested seed and plant material, must be maintained by the applicant and made available to the CFIA upon request.

A complete description of trial terms and conditions are available on the CFIA website and are updated annually (CFIA 2009d).

Each confined research field trial authorized by the CFIA is inspected multiple times to verify compliance with the terms and conditions of the authorization. CFIA inspectors are trained and certified to conduct these inspection activities. If an inspector discovers any instance of non-compliance, actions must be taken to immediately return the situation to compliance, up to and including the termination of the trial and destruction of the crop if necessary. CFIA inspectors also examine trial sites in subsequent growing seasons when the sites are subject to post-harvest land use restrictions. Inspectors verify that the applicant is properly monitoring the site and removing volunteer plants, as well as keeping appropriate records and maintaining any harvested material with appropriate labeling and in contained conditions.

Trial applicants have, in general, met the terms and conditions imposed by the CFIA on each of these trials. The CFIA has a performance target of at least 90% of trials operating in compliance with terms and conditions, with 100% of trials returned to compliance when infractions occur (CFIA 2009a). Recognizing that there may be a level of uncertainty about the environmental interactions of PNTs growing in confined field trials, a cautious and thorough approach is taken when developing terms and conditions and verifying compliance.

9.4.2 Unconfined Release

If a product developer wishes to commercialize a PNT, or cultivate it on a larger scale than permitted within the confined field trial program, he or she may apply for unconfined environmental release. Before PNTs may be authorized for unconfined release in Canada, they must undergo an environmental safety assessment. As mentioned previously, in the early 1990s AAFC conducted a series of consultations with a wide range of stakeholders to determine the most appropriate information requirements

for applications for unconfined release. These requirements address three issues: the precise characterization of the PNT, the relative phenotypic expression of the novel traits, and the potential interactions of the PNT in the environment. Guidelines specific to unconfined release applications were drafted to assist applicants with the interpretation of the general data requirements outlined in Part V of the Seeds Regulations. The original Directive 94–08, Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits, was first published in 1994. In order to keep pace with the most recent science as well as experience gained by regulators, the document was revised in 2000, and then again in 2004 (CFIA 2004b).

Given the wide spectrum of PNTs for which an unconfined environmental release authorization may be sought, information requirements listed in Directive 94–08 may not be relevant for every PNT submitted for review. Applicants may address certain information requirements listed in the Directive by providing a valid written scientific rationale in lieu of experimental data (CFIA 2009b).

Key to the environmental assessment approach of the CFIA is the development of biology documents for each crop species for which a PNT was intended for unconfined release. These documents provide baseline information about the agricultural, agronomic and environmental behaviour of each crop species in the Canadian environment, e.g., details on centres of origin, the potential for gene introgression into related species, other environmental interactions, and how and where the crop is cultivated in Canada. Biology documents provide a backdrop for the environmental assessment of the PNT by assisting with the assessment of whether the PNT's novel gene products could potentially cause the PNT to become a weed of agriculture, become invasive of natural habitats, or be otherwise harmful to the environment, relative to an unmodified counterpart.

The CFIA's approach of using biology documents to aid an environmental safety assessment has been influential internationally. The Working Group on Harmonization of Regulatory Oversight in Biotechnology of the Organisation for Economic Development (OECD) prepares similar "consensus documents" that describe the biologies of key crop plants from a broader geographical perspective.

In meeting the extensive information requirements for applications to the CFIA for unconfined release, applicants are likely to have conducted prior experiments, including at the confined release stage. These experiments are expected to contribute data which will address the five key criteria of environmental safety assessments:

- Potential for the PNT to become a weed of agriculture or invasive of natural habitats
- Potential for gene flow to wild relatives whose hybrid offspring may become more weedy or more invasive
- Potential for the PNT to become a plant pest or increase the activity of a pest
- Potential impact of the PNT or its gene products on non-target species, including humans
- Potential impact on biodiversity.

The generation of data to be considered for a determination of environmental safety must be produced using statistically valid experimental designs and protocols

(i.e., equivalent to the standards required for inclusion in peer-reviewed research publications). Typically this information is based largely on data generated in experiments at the confined release stage in Canada. However, information required for an unconfined release submission need not exclusively come from such experiments; existing information in peer reviewed scientific literature and data from similar environments in other jurisdictions may also be used. CFIA officials also consult external experts or other pertinent information generated from the Agency's own research on specific key environmental areas. If an application for unconfined environmental release is found to have any deficiencies in the data required to arrive at a determination of safety, additional information or studies will be requested from the product developer.

Once a comparative environmental safety assessment of the PNT has been completed, the CFIA considers whether risk management measures are required to address any issues identified in the course of the safety assessment. All PNTs authorized will be subject to a condition that any new information obtained by the product developer regarding the safety of the PNT must be supplied to regulatory authorities. If the CFIA receives such information, it is evaluated and the terms of the authorization may be adjusted if warranted (up to and including withdrawal of the original authorization if that were to be deemed necessary). In addition to this general condition, certain types of PNTs have been authorized with conditions that support their sustainable use in the long term. Since 2004, PNTs with novel herbicide resistance traits have been authorized with conditions that help ensure farmers using these crops are provided information on volunteer management and crop and herbicide rotation to delay the development of resistant weeds. Insect-resistant Bt corn products are authorized conditional on the use of stewardship plans that aim to delay the development of resistant insects. In general, the CFIA has the ability to apply conditions on the authorization of the unconfined release of any PNT, in order to manage environmental risks relative to their conventional counterparts.

At the time of writing, 64 PNTs have been assessed and authorized for unconfined release in Canada. The majority of these authorized PNTs have novel herbicide resistance traits or Bt traits conferring resistance to insect pests. Other traits include viral disease resistance and altered oil quality profiles. The predominant crop species involved include canola, corn, soybeans and potato. While the majority of these PNTs were derived through rDNA techniques, some were derived through other traditional plant breeding methods. A database of PNTs approved for environmental release in Canada is maintained on the CFIA web site (CFIA 2010). Recognizing that other jurisdictions apply a process-based (rDNA) regulatory trigger, for purposes of transparency, this database indicates which PNTs authorized in Canada also meet the definition of LMOs under the Cartagena Protocol on Biosafety.

Once authorization for unconfined environmental release has been given, the PNT may still be subject to other regulatory processes prior to its commercial use. Canada employs a "no split approval" policy, that aligns the timing of regulatory decisions for those PNTs which are also novel foods and novel livestock feeds. For crop kinds that are subject to variety registration in Canada, PNTs, novel foods and novel feeds must have their authorizations for environmental release, use in food

and feed in place before these varieties can be registered. Ultimately, a decision to commercialize or to discontinue a PNT in Canada is made by its developer. Several of the PNTs which have been approved for unconfined environmental release in Canada are no longer (or never were) in commercial production.

9.4.3 Stacked Products

Stacked products can be defined as conventional crossing of two or more authorized products to produce a combined trait product. A 2009 report from the Joint Research Centre of the European Commission indicates that hundreds of potential new "stacks" will be possible by 2015, although the commercial viability of these products was not evaluated (Stein 2009).

Canadian biotechnology regulators are increasingly asked about Canada's approach to regulating stacked products. The simple answer is that concerns related to stacking can be largely ruled out during the initial assessments of the parental lines. However, as a precaution, the CFIA requires notification of all stacked products before they are introduced into the marketplace. From an environmental safety perspective, the main considerations are whether the stewardship conditions applied to the parental lines are still appropriate for the stacked product (CFIA 2009e).

As described previously, the environmental safety assessments of PNTs take into account a range of data that are outlined in detail in the guidance documents. Among these requirements is information to demonstrate that the novel trait is stably inherited and that it has not caused unintended effects on the agronomy or composition of the plant. Another requirement is an analysis of any potential impacts of gene flow, or crossing of the PNT with other plants that may occur once the PNT is released.

Based on the assessment of these factors, the authorization of a PNT for unconfined environmental release includes a permission to use the PNT in breeding programs to develop new varieties that will also contain the novel trait. Thus, an individual PNT authorized for unconfined environmental release, may be used to develop many different varieties that are regionally or otherwise adapted to meet the needs of farmers. Similarly, many stacked products do not require further assessment of their environmental safety prior to their environmental release in Canada, and are exempted from the requirements of confined environmental release described previously.

This approach recognizes that the environmental safety assessments conducted on the parental lines have already taken into account many of the considerations that establish the safety of a stacked product. However, notifications are required so that regulators may determine if any conditions of an unconfined release authorization placed on the parental PNTs are also appropriate for the stack, and whether additional information is required. Cases in which alterations to stewardship conditions are proposed (e.g. altered refuge requirements for Bt corn products), or if the stacked product expresses an additional novel trait which is not present in the parental lines, could require additional information and analysis (CFIA 2009e).

9.5 Future Challenges

The increasing complexity of products of biotechnology in development, whether resulting from further stacking of familiar traits or the introduction of new traits, such as those for drought resistance, nutritional enhancement, or production of industrial or pharmaceutical products, will require efficient and nimble regulatory processes. This will allow the benefits of new products, whether of conventional or biotechnology, ever more important in the face of climate change and rising threats to food security, to be realized in a manner that protects the environment and biodiversity. Many of these benefits may be realized outside of North America, as over the next 5 years we will see the predicted shift away from Canada and the United States as the primary locations of the developers and initial adopters of new biotech crop varieties.

As the number of products of biotechnology in the global marketplace grows, as more jurisdictions implement domestic regulatory regimes, and as detection methods increase in their availability and sensitivity, the potential for asymmetric product authorizations between trading partners will continue to grow. Additionally, some countries which have been traditional importers of biotech products produced elsewhere are on the cusp of becoming producers and exporters themselves. Likewise, countries which have been primarily exporters will soon have the opportunity to import products of biotechnology from a greater number of sources. Accordingly, the trade disruptions that have resulted when low level presence of products of biotechnology are unexpectedly detected in agri-food commodities may worsen.

In Canada, as in many other jurisdictions around the world, the presence of an unauthorized product of biotechnology in the marketplace or environment constitutes regulatory non-compliance. Over the last decade, there have been a handful of occurrences of unapproved products which have entered the Canadian environment in the absence of authorization, typically at low levels. All of these occurrences have been returned to compliance through efforts to remove the non-compliant product from production, or through the authorization of the product through appropriate regulatory processes.

There have also been situations in which products that are fully authorized for unconfined environmental release in Canada have been detected in other jurisdictions where the product is not (yet) authorized, resulting in regulatory issues and trade disruptions.

When an asymmetric product authorization leads to a situation of regulatory non-compliance, the impacts on the trade of agricultural commodities can be severe, and may be disproportionate to the risk (if any) posed by the situation. When low level presence of a product leads to a trade disruption, the impacts may be more far-ranging than expected, with unwitting exporters, importers, farmers and end users of a commodity being negatively affected. This is an unfortunate occurrence, despite a growing body of evidence confirming that products of biotechnology are no more likely to pose risks than the products of other plant breeding techniques.

Various approaches to manage low level presence are now being discussed in a variety of forums, including the OECD and the Asia Pacific Economic Cooperation, as well as bilaterally between trading partners. In the long term, movement towards universal data packages, common or harmonized risk assessments, and international or regional regulatory decisions could help to reduce the potential for asymmetric product authorizations to occur, notwithstanding the right of countries to make sovereign decisions.

In addition to avoiding regulatory non-compliance issues, these types of harmonized approaches could also help to make the global regulatory system for biotechnology less onerous on the deployment of products developed by public research institutions or small and medium sized enterprises. Currently, the costs associated with obtaining and maintaining regulatory authorizations for a new product in all of the major markets that it could enter at low or unintended levels are prohibitive to these developers.

In light of global issues such as climate change and food security, it will become increasingly necessary and proper for regulatory authorities to evaluate the level of regulatory burden applied to products of biotechnology, given that the body of research supporting the safety of these products continues to mount, as do opportunities to use them for the public good.

References

CEPA (1988) Canadian Environmental Protection Act (S.C. 1988 c. 22)

CEPA (1999) Canadian Environmental Protection Act (S.C. 1999, c.33) Retrieved 15 Aug 2010, from http://laws-lois.justice.gc.ca/eng/acts/C-15.31/index.html

CFIA (Canadian Food Inspection Agency) (2004a) Biotechnology notices of submission. Retrieved 15 Aug 2010, from http://www.inspection.gc.ca/english/plaveg/bio/subs/subnote.shtml

CFIA (Canadian Food Inspection Agency) (2004b) Assessment criteria for determining environmental safety of plants with novel traits. Retrieved 15 Aug 2010, from http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9408e.shtml

CFIA (Canadian Food Inspection Agency) (2009a) Canadian food inspection agency 2008–2009 performance report. Retrieved 15 Aug 2010, from http://www.tbs-sct.gc.ca/dpr-rmr/2008-2009/inst/ica/ica00-eng.asp

CFIA (Canadian Food Inspection Agency) (2009b) Directive 2009–09: plants with novel traits regulated under Part V of the seeds regulations: guidelines for determining when to notify the CFIA. Retrieved 15 Aug 2010, from http://www.inspection.gc.ca/english/plaveg/bio/dir/dir0909e.shtml#a4

CFIA (Canadian Food Inspection Agency) (2009c) Directive Dir 2000–07: conducting confined research field trials of plant with novel traits in Canada. Canadian Food Inspection Agency, Ottawa

CFIA (Canadian Food Inspection Agency) (2009d) Plants with Novel Traits (PNTs) – approved confined research field trials/terms and conditions. Retrieved 15 Aug 2010, from http://www.inspection.gc.ca/english/plaveg/bio/confine.shtml

CFIA (Canadian Food Inspection Agency) (2009e) Regulating the environmental release of stacked plant products in Canada. Retrieved 15 Aug 2010, from http://www.inspection.gc.ca/english/plaveg/bio/staempe.shtml

CFIA (Canadian Food Inspection Agency) (2010) Plants evaluated for environmental and livestock feed safety. Retrieved 15 Aug 2010, from http://active.inspection.gc.ca/eng/plaveg/bio/pntvcne.asp

Environment Canada (2001) Regulatory roadmap for new substances in Canada. Retrieved 15 Aug 2010, from http://www.ec.gc.ca/subsnouvelles-newsubs/default.asp?lang=En&n=157FDFE2-1

Government of Canada (1993) Federal government agrees on regulatory framework for biotechnology. Retrieved 15 Aug 2010, from http://www.bioportal.gc.ca/English/LinkSearch.asp?x=1&formAction=ShowItemDetails&ID=633&DeptID=&bioreg=

Government of Canada (1996) Part V release of seed. Regulations respecting the quality of seeds including seed potatoes, and the testing, inspection, and sale thereof (C.R.C., c. 1400)

Government of Canada (1998) The 1998 Canadian biotechnology strategy: an ongoing renewal process. Retrieved 15 Aug 2010, from http://www.bioportal.gc.ca/English/view.asp?x=535&mid=24

Government of Canada (2001) Action plan of the government of Canada in response to the royal society of Canada expert panel report. Retrieved 15 Aug 2010, from http://www.hc-sc.gc.ca/sr-sr/pubs/gmf-agm/RSC_response-reponse_SRC-eng.php

James C (2009) Global Status of Commercialized Biotech/GM Crops: 2009. ISAAA Brief No. 41. ISAAA: Ithaca, NY

National Academy of Sciences (1989) Field testing of genetically modified organisms: framework for decisions. National Academy Press, Washington, DC

National Forum on Seed (2008) Facilitating broad consultation and consensus building in the Canadian seed and crop sectors. Retrieved 15 Aug 2010, from http://www.nationalforumonseed.com/Meetings/meetings-e.html

OECD (1986) Recombinant-DNA safety considerations. OECD, Paris

Stein AJC (2009) The global pipeline of new GM crops implications of asynchronous approval for international trade. European Communities: European Commission Joint Research Centre, EUR 23496 EN

The Royal Society of Canada (2001) Elements of precaution: recommendations for the regulation of biotechnology in Canada. Royal Society of Canada, Ottawa

Chapter 10
EPA Regulatory Requirements for Plant-Incorporated Protectants

John L. Kough and Rebecca Edelstein

Abstract This chapter provides a discussion on EPA regulation of pest control traits expressed in living plants, termed Plant-Incorporated Protectants (PIPs). These are regulated as pesticides and, as such, require assessment by EPA prior to field testing and commercialization. This chapter provides a discussion of EPA regulatory requirements for PIPs as well as a description of the data that EPA typically uses to make safety determinations.

Keywords Plant-Incorporated Protectant (PIP) • Pest control • Pesticide • EPA • Regulation • Risk assessment • Ecological risk assessment • Insect resistance management • Field testing • Experimental use permits • Federal Insecticide, Fungicide and Rodenticide Act • Federal Food, Drug, and Cosmetic Act

10.1 Introduction

A pest control trait expressed in living plants is termed a Plant-Incorporated Protectant (PIP), which is defined in the Code of Federal Regulations 40 (CFR) 174.3 as a "pesticidal substance that is intended to be produced and used in a living plant, or in the produce thereof, and the genetic material necessary for the production of

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such a pesticidal substance." PIPs fall within the definition of a pesticide in the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) section 2(u) which states that a pesticide is a substance or mixture of substances intended to prevent, destroy, repel or mitigate a pest. A pesticide also includes plant growth regulators, defoliants and desiccants and nitrogen stabilizers.

The Environmental Protection Agency (EPA) is responsible for regulating the sale, distribution, and use of pesticides to protect human health and the environment. FIFRA section 3(a) requires, with some exceptions, that a pesticide be registered under the Act prior to distribution or sale in the United States. To register a pesticide, EPA evaluates the proposed pesticide to ensure that its use will not pose an unreasonable risk to human health or the environment. Under FIFRA section 5, EPA issues Experimental Use Permits (EUPs) to allow prospective registrants to generate information or data necessary to register a pesticide. In addition, the Federal Food, Drug, and Cosmetic Act (FFDCA) authorizes EPA to establish tolerances (maximum limits) or exemptions from the requirement of a tolerance for residues of pesticides in food. Under FFDCA, food that contains pesticide residues is considered adulterated and subject to seizure by the Food and Drug Administration unless EPA has issued a tolerance exemption or a tolerance (and the residue is within the limits). Regulatory requirements, criteria, and procedures applicable to PIPs are outlined in 40 CFR 174 and 40 CFR 152.

All plants have defense mechanisms to prevent or mitigate damage from herbivorous pests and diseases. Many of these pest defenses are based on the induced production of protective compounds or the activation of preformed compounds into pest deterrents. All these naturally occurring plant defense compounds can also be considered pesticides under the definition found in FIFRA. The pest control compounds found in plants produced by conventional breeding techniques have a significant history of safe use in the diet and the environment. Because of this history of safe use, EPA has exempted from FIFRA requirements (except for the requirement to report adverse effects that are the result of these pest resistance traits) and tolerance requirements under FFDCA, products of conventional breeding that may technically fall under the pesticide definition.

10.2 EPA Risk Assessment of PIPs

10.2.1 General Considerations

To determine whether to register a PIP under FIFRA or issue a tolerance or tolerance exemption under FFDCA, EPA conducts risk assessments of PIPs. The main focus of EPA's PIP risk assessments has been on pest resistance traits introduced through genetic transformation because these represent new traits and possibly new exposures. The PIPs registered to date have provided crop protection from insect damage and virus infection. The current PIP traits are based on expressing a protein

insect toxin or triggering inherent virus resistance pathways. EPA does not register a transgenic plant itself but rather a PIP expressed in a plant. The plant dictates the issues related to environmental exposure and possible movement of the PIP genetic material into related plants. The PIP risk assessment examines the hazards of the PIP as an isolated pesticidal substance and PIP expression in the transformed plant to evaluate potential exposure to the pesticidal substance.

Companies generate data that EPA uses in the risk assessment to support a pesticide registration and, if needed, a tolerance exemption. EPA is required to make separate determinations for a pesticide registration and for a tolerance or tolerance exemption for a food use of a pesticide. EPA issues a registration under FIFRA, with a safety standard of no unreasonable adverse effects to the environment with the pesticide's use. EPA conducts dietary evaluations under FFDCA with the safety standard of a reasonable certainty of no harm to the aggregate exposure to the pesticide residues. Under FFDCA, EPA is specifically tasked with setting the tolerance levels (i.e., maximum residue limits) for pesticide residues that may occur in or on treated foods. If EPA determines that there will be no harm from any reasonably expected level of residues from the PIP, then EPA can establish an exemption from the requirement for a tolerance.

10.2.2 Data Requirements

To date, the information that companies have generated to support these decisions has been based on the data required to register a microbial pesticide (40 CFR 158 subpart V) and product details needed for a notification of an experimental release of a genetically modified microbial pesticide (40 CFR 172 subpart C). EPA has used a case by case approach to evaluate PIPs, with different data being required for different PIPs. The data requirements that address hazard endpoints for microbial toxins (e.g., oral toxicity, honeybee toxicity) have been most useful since most PIPs are based on insect toxins from *Bacillus thuringiensis.* While this case by case approach has worked for products seen to date, EPA has decided a more complete and specific set of data requirements for the risk assessment of PIPs should be provided to clarify the regulatory expectations for companies developing products. With this in mind, there is currently an ongoing effort to formalize specific data requirements for PIPs.

The next sections of this chapter provide some discussion of the data that has been evaluated by EPA for the risk assessments of PIPs as well as the rationale behind the chosen endpoints examined to justify a PIP pesticide registration and/or tolerance decision. The basis for oversight is that agricultural biotechnology allows for the introduction of genetic traits from a much wider spectrum of sources than are available from conventional breeding. The technology also allows for protein engineering and the development of entirely new proteins with activities that may not occur in nature. There are technological limitations to what can be done and constraints on protein structure that may limit what can actually be functionally expressed *in planta*. While not exhaustive, there are currently three types of genetic

manipulations in plants nearing commercial marketing or that are being developed as PIPs: new proteins; new biosynthetic pathways; and small RNAs triggering inherent interference mechanisms. These three types of genetic manipulations raise some distinctive regulatory questions which will be referred to as the different data requirements are discussed.

10.2.3 Product Characterization

Product characterization is an important component of the risk assessment of PIPs. Product characterization information evaluated by EPA includes several types of data such as details of the plant transformation process, the DNA used in transformation, the DNA introduced into the plant genome, and expression of the PIP trait. Further information consists of the stability of the trait over time such as several breeding generations or vegetative propagation cycles. For longer lived plant species this may entail evaluating growth and PIP expression over several growing seasons. The information can consist of either tracking the DNA of the trait itself or looking for the phenotypic expression of that trait if it can be readily determined.

The product characterization also contains a detailed account of the introduced PIP trait. Besides the details of the PIP DNA construction, the transformation process, and what DNA is actually incorporated into the genome, the product characterization typically has a discussion of the expected phenotype of the transformed plant. Proteins represent the most common type of trait currently transformed into plants and therefore the greatest variety of these traits has been developed by commercial interests. The expression of a single toxin protein to give an insect resistant phenotype fits easily into the current PIP regulatory scheme for a simple gene (e.g., promoter, coding sequence and terminator) being responsible for a single protein output. A single protein from a single gene also reflects the current central dogma of biology: the DNA from a gene is transcribed into messenger RNA and then translated into a protein.

There are other possible PIP types, mentioned above, that do not follow the central dogma. Some of the virus resistant plants such as the potato expressing the potato leaf roll virus (PLRV) replicase gene expressed a phenotype without any apparent protein output from the introduced gene (U.S. EPA 2000). The activity of this PLRV resistant PIP product was subsequently ascribed to the phenomenon of RNA interference. Similar to research being pursued in pharmaceuticals, protein engineering can alter the amino acid (AA) sequence of a protein to produce a PIP protein with new activities, not previously demonstrated to exist in nature. Metabolic engineering, with introduction of enzyme pathways for the production of low molecular weight PIP compounds, may present new exposures in the host plant from both the enzyme protein(s) and the low molecular PIP compound itself. With greater knowledge of plant responses to injury by pests, there is also the possible induction of inherent plant defense pathways that would have a faster response to injury thereby preventing pest damage. All of these different types of PIPs could result in either the production of substances not previously produced in the plant or

the suppression of an existing plant function. An important component of product characterization therefore also includes describing the new substance(s) (if any) produced in the plant.

10.2.4 Exposure Assessment (Plant Biology and Gene Flow)

A critical component for the PIP risk assessment is determining the potential for environmental and dietary exposure to the PIP presented by the transformed plant. It is critical to understand that EPA does not regulate the transformed plant itself but rather the PIP introduced into that plant. However, EPA does assess potential exposure to the PIP by considering information, including that submitted by the developer, on the basic biology of the plant, including a discussion of the taxonomy of the species and its relatives, the genetics and potential for natural hybridization, flowering phenology, climatic requirements, and the cultivation and ecology of the plant. Information on the extent of crop cultivation, the potential for trait hybridization and introgression, and potential for expression in wild plant relatives is also used for evaluating potential exposure to the PIP. To date, the plant species transformed with PIPs have been mostly corn, cotton, potato, several other vegetables and, more recently, a perennial fruit tree, plum. Therefore, for these few well known species, the information on host plant biology has been readily available. In the future, it is possible that the risk assessment process may be delayed as details of plant ecology are investigated to properly frame the issues of PIP exposure and gene flow potential. Fortunately, the Organization for Economic Cooperation and Development has been generating numerous plant biology consensus documents that consolidate the pertinent scientific literature and provide the background information, which facilitates this aspect of the risk assessment process (OECD 2006).

10.2.5 Hazard Assessment

Once the PIP trait and the transformed plant have been adequately described, the data and tests needed to make a safety finding under FIFRA and, if necessary, FFDCA can be determined. If the transformed PIP expressing plant is producing a new protein, and the plant is a food crop, there is a process to follow as found in the Codex Alimentarius (2003). The basis of this assessment is the source of the protein trait, AA sequence similarity comparison, and stability to pepsin and heat. For PIP proteins, there is the presumption that the potential for toxicity exists (since the protein is toxic to the pest) unless information on mode of action, target pest specificity or other information indicates the contrary. The safety of naturally occurring nucleic acids has been affirmed by both the U.S. Food and Drug Administration's (U.S. FDA's) statement that there are no safety issues associated with nucleic acids introduced into new plant varieties (U.S. FDA 1992) and for PIPs specifically, by a

tolerance exemption (40 CFR 40 174.507). The safety of other PIP compounds, which are not nucleic acids or proteins, should be assessed based on known prior exposure, similarity to chemically related compounds or other information on a case by case basis.

EPA assesses hazard of the PIP by considering possible outcomes from the transformation events. In the simplest cases, EPA assesses the hazards from the newly expressed protein(s) based on knowledge of the mode of action and specificity of the PIP trait and the responsible pesticidal protein. Developers of PIPs generally provide information on the mode of action by demonstrating pesticidal activity against the target pest with a theoretical discussion of the PIP's site of activity in that pest. In some cases, verification of this site is demonstrated with histological examination of tissue disruption or binding and inactivation of specific metabolic functions in the affected pest. Specificity of the PIP trait to a limited number of species can be initially shown with a challenge of the PIP pesticidal substance (isolated or expressed *in planta*) to numerous pest species and a limited range of pests being effectively controlled. This information frames the additional non-target tests that may be needed to complete an environmental risk assessment. For other scenarios where an isolated pesticidal substance is not easily obtained or an inherent pathway is triggered, the demonstration of mode of action may be problematic and more than likely derived from information on plant phenotypic expression under different pest pressures or challenges.

The hazard analysis of proteins has been facilitated by the development and use of bioinformatic analysis of protein structure. These bioinformatic analyses are based on algorithms (e.g., BLAST) that compare the PIP protein amino acid (AA) sequence(s) and the ever expanding databases of known protein sequences. These analyses provide a powerful means to discover relationships among proteins, infer function and suggest potential hazard from just the AA sequence. Moreover, *in silico* techniques can reduce the use of animal testing in the hazard assessment process. As this computer based analysis develops, analysis of secondary and tertiary structure will likely be involved, leading to further refined predictions from *in silico* methods (Ivanciuc et al. 2003). Currently the bioinformatics analysis is used to establish relationships between the PIP protein and any potentially hazardous proteins like toxins, anti-nutritional factors and allergens. This AA similarity analysis has been accepted in the Codex Alimentarius to trigger possible further testing for those proteins that show significant similarity to hazardous proteins, especially allergens. Similarity to a known or recognized allergen protein suggests that further testing is needed, such as specific serum testing. The similarity is defined as 35% identity over an 80 AA sequence comparison or a 6–8 AA stepwise analysis match between the PIP protein sequence and any allergen AA sequence (Codex 2003).

If a determination is made that dietary safety data is needed for a protein PIP, potential oral toxicity is considered. As most proteins are larger molecules and have limited absorption by other exposure routes (Guy and Hadgraft 1991), the primary exposure for consideration in mammalian toxicity is the dietary route. The toxicity test suggested is an acute oral toxicity study in the rodent with purified PIP protein at or near the limit dose of 2–5 g/kg bodyweight (U.S. EPA 2002). The acute oral toxicity study helps

to determine whether PIP proteins behave like any other proteins in an oral exposure. The protein should breakdown under gastrointestinal exposure to digestive enzymes, be absorbed like a typical dietary protein and display no adverse effects after consumption. Toxic proteins, however, are known to display adverse physiological effects with an acute exposure at high doses (Sjoblad et al. 1992). The intent of the acute oral toxicity test is to verify that the PIP protein is similar to other dietary proteins and does not cause significant adverse effects with oral exposure.

For the acute oral toxicity study or any mammalian toxicity test for PIP proteins, the test substance should be purified protein. The use of a purified protein dosing preparation for any required tests is important because EPA is evaluating the safety of the PIP itself, not the whole plant. Use of purified protein as the test substance rather than whole plant material allows EPA to isolate the effect of the PIP protein from the effect of the rest of the plant matrix on the test animal's diet and health. The use of purified material at a high dose also justifies a reasonable certainty of being able to make a safety finding with appropriate margins of exposure employing the results of the test. The amount of pure protein needed to perform even a limited toxicity test on rodents is in the amount of several grams of purified material. With plant expression levels in most tissues in the ppm concentration range, it is frequently not feasible to purify enough protein from plant tissue. Therefore, companies often produce the PIP protein test substance in an alternate production system such as a yeast or bacterium. When an alternate production system is used, the microbially produced protein (i.e., alternate test substance) must be shown to have similar biochemical characteristics and bioactivity to that produced in the PIP expressing plant. The characterization data for the alternate test substance is compared to that generated for the PIP as expressed in the plant. It is essential that the plant produced PIP be included as a reference material in the characterization tests done to verify the utility of the alternate test substance for toxicity testing.

10.2.6 Ecological Risk Assessment

EPA conducts an ecological risk assessment to establish that the PIP as expressed in a given plant can be used without presenting an unreasonable adverse risk to the environment. The basis for making this determination is similar to that done for making a human health safety finding. The appropriate information or data to make the determination for a PIP can be done with surrogate animal testing, literature citation, similarity to chemically related compounds, knowledge of prior environmental exposure and other clarifying data on a case by case basis. Much of this environmental hazard data and information is framed by a thorough discussion of the expected exposure anticipated with deployment of the PIP expressing plant, the plant's biology, and information about the presence of compatible wild relatives in the area of cultivation. The overall determination is based on both the potential hazard of the PIP and the expected exposure. If there is no demonstrated hazard from the PIP, exposure to a PIP expressing plant itself is not a risk endpoint.

The array of surrogate animals used for testing to establish safety for non-target organisms in the environment are intended to represent a reasonable selection of those animal and plant orders similar to those that are expected to be exposed to the PIP. The data requirements for microbial pesticides provide the best examples of which surrogate animal species have been used for testing (40 CFR158.2150), and the guidelines for how to perform the tests provide a rationale for choosing the species to be tested (U.S. EPA 1996). While there are some significant differences between microbial agents and plants expressing PIPs (there are no pathogenicity concerns for a plant expressed PIP trait), species selection is still based on expected exposure from use of the pesticide. In addition, the species that should be used for testing is sometimes altered if the PIP characterization data indicates that a specific group of species would be more appropriate surrogates than those typically employed. One example of this has been the use of predatory ground beetle species as a test species to assess the potential of coleopteran specific Cry toxins to affect non-target insects (U.S. EPA 2010a, b). These alterations reflect the case by case nature of the PIP risk assessment process and how refinements can improve the suitability of the data used to perform the environmental risk assessment.

If there is an identified environmental hazard and the PIP expressing plant has the ability to form viable progeny with wild related species in the vicinity of the cultivated crop, consideration of the potential for movement of the trait into wild plant populations by introgression and possible effects of the PIP pesticidal traits in the wild populations would need to be considered. Movement of the PIP trait into other plant populations is considered biological fate of the trait. The possible environmental risk is contingent on the verification that the PIP expressing plant and the wild relative are capable of forming fertile progeny and that the PIP trait could introgress into the wild population. For introgression into the wild population to occur, there would probably have to be selection pressure from a PIP controlled pest present that was significantly affecting the wild relative population.

There is also the potential for chemical residues of the PIP pesticidal substance to persist in the environment and potentially affect resident populations of non-target species. Even though functional proteins are expected to have a limited lifetime in the environment due to microbial degradation, some studies suggest that Cry proteins bound to clay particles in the soil could affect that assumed instability (Tapp and Stotzky 1998; Saxena and Stotzky 2000). PIP pesticidal substance environmental stability is examined to determine if there are possible long term effects on susceptible populations. For the PIP Cry proteins that have been examined to date, this analysis has entailed testing of soil and crop residues for their activity against the target pest. The initial assays were done in isolated soil samples with introduced proteins (U.S. EPA 2001). These studies have been supplemented with others done on soils that have been under continuous cultivation with PIP expressing crops for a number of years (Sanvido et al. 2006). To date, no unusual persistence has been seen in these soil studies as would be expected for proteins in general. The suggestions from controlled laboratory studies that under certain sterile soil conditions Cry protein stability may be enhanced due to clay particle adsorption has not been confirmed by

the field study results. Therefore, EPA has concluded that enhanced stability and buildup over continuous cultivation of PIP expressing plants is not a concern for those plants expressing Cry proteins.

10.3 Insect Resistance Management

EPA also has circumstances where additional data may be needed to address the development of pest resistance. The PIPs registered as of 2011 have a demonstrated lack of significant adverse effects to both the environment and human health. Because these PIPs have such low risks and have successfully reduced the use of potentially more hazardous conventional pesticides, EPA has determined that it is in the public's interest to ensure that the use of PIP crops expressing Cry proteins have an extended utility and that their deployment does not adversely affect the use of microbial products important in organic crop production. EPA has been involved with the companies registering PIPs to implement programs aimed at reducing the likelihood the targeted pests develop resistance to the PIP trait. Similar to the situation in the deployment of antibiotics in the clinical realm or new chemistries for pesticide active ingredients, an understanding of the potential for target organism to develop resistance to the PIP trait from selection pressure is essential. To this end EPA has published a program of points to address for implementing effective resistance management plans. As with all the issues related to PIP expressing plants, there have been numerous Science Advisory Panels to help EPA in compiling the appropriate science for generating guidance. The resistance management plans developed to date are described in detail for several products (U.S. EPA 2006a, b).

10.4 Field Testing Using Experimental Permits

USDA and EPA have concurrent jurisdiction over the field testing of genetically engineered plants containing PIPs. USDA requires a notification, or permit prior to the field testing or the introduction into the environment of any regulated article (USDA 2008, 2010). In general, a new genetically engineered plant, including a plant containing a PIP, is considered a regulated article. EPA requires an Experimental Use Permit (EUP) to field test an unregistered PIP or an unregistered use of a PIP on a cumulative total of over 10 acres. Under FIFRA section 5, EPA issues EUPs to allow prospective registrants to generate information or data necessary to register a pesticide. If any residues of the PIP may be anticipated to enter the food supply during field testing, even through pollination of adjacent crops, the sponsor of the field test is required to request a temporary food tolerance determination (U.S. EPA 2007). General EUP application requirements are codified at 40 CFR. § 172.4. In addition, EPA held a 2-day public workshop on EUPs for PIPs in February 2004 and has published the workshop's proceedings, including questions and answers

from industry, as guidance to PIP EUP applicants (U.S. EPA 2004). EPA also provides suggestions for how to calculate the area for a PIP EUP on its website (U.S. EPA 2011a).

10.5 Conclusions

The discussion of PIP data requirements provided above is an overview of the approach used by EPA to make safety determinations under FIFRA and FFDCA. While the discussion attempts to cover several anticipated PIP product types, the best descriptions of the process used to date for each of the registered PIPs are the actual risk assessment/decision documents, which can be found at the EPA website (U.S. EPA 2011b). These documents provide an accurate rendition of the data and assessment used to make a safety determination and thoroughly describe the assessment process such that the reader can anticipate how EPA may conduct future case by case assessments of newer PIP products.

References

40 CFR 158 subpart V. Code of federal regulations title 40: Protection of environment, Part 158—Data requirements for pesticides, Subpart V—Microbial pesticides. http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=46d22bc6e9a74d8f268ee0ee49f89201&rgn=div5&view=text&node=40:24.0.1.1.9&idno=40#40:24.0.1.1.9.16. Accessed June 27, 2012

40 CFR 158.2150. Code of federal regulations title 40: Protection of environment, Part 158—Data requirements for pesticides, Subpart V—Microbial pesticides, 158.2150—Microbial pesticides nontarget organisms and environmental fate data requirements. http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=46d22bc6e9a74d8f268ee0ee49f89201&rgn=div5&view=text&node=40:24.0.1.1.9&idno=40#40:24.0.1.1.9.16.1.6. Accessed June 27, 2012

40 CFR 172 subpart C. Code of federal regulations title 40: Protection of environment, Part 172—Experimental use permits, Subpart C—Notification for certain genetically modified microbial pesticides. http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=46d22bc6e9a74d8f268ee0ee49f89201&rgn=div5&view=text&node=40:24.0.1.1.22&idno=40#40:24.0.1.1.22.3. Accessed June 27, 2012

40 CFR 174.507. Code of federal regulations title 40: protection of environment, Part 174—Procedures and requirements for plant-incorporated protectants, Subpart W—Tolerances and tolerance exemptions, 174.507—Nucleic acids that are part of a plant-incorporated protectant; exemption from the requirement of a tolerance. http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=46d22bc6e9a74d8f268ee0ee49f89201&rgn=div8&view=text&node=40:24.0.1.1.24.14.19.7&idno=40. Accessed June 27, 2012

Codex Alimentarius (2003) Foods derived from biotechnology. Alinorm 03/34: joint FAO/WHO food standard programme, Codex Alimentarious Commission, twenty-fifth session, Rome, Italy, 30 June–5 July 2003. Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants, and appendix IV, Annex on the assessment of possible allergenicity, pp 47–60

Guy RH, Hadgraft J (1991) Principles of skin permeability relevant to chemical exposure. In: Hobson DW (ed) Dermal and ocular toxicology: fundamentals and methods. CRC Press, Boca Raton

Ivanciuc O, Schein CH, Braun W (2003) SDAP: database and computational tools for allergenic proteins. Nucleic Acids Res 31:359–362

OECD (2006) Organisation for Economic Co-operation and Development (OECD) (2006) Series on harmonisation of regulatory oversight in biotechnology, no. 35, points to consider for consensus documents on the biology of cultivated plants. Environment directorate. Paris. Available online at http://www.oecd.org/dataoecd/36/17/46815838.pdf. Accessed June 27, 2012

Sanvido O, Stark M, Romeis J, Bigler F (2006) Ecological impacts of genetically modified crops, experiences from ten years of experimetnal field research and commercial cultivation. Agroscope Reckenholz-Tanikon Research Station ART, Zurich, 108 p

Saxena D, Stotzky G (2000) Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic *Bt* Corn in vitro and in situ. FEMS Microbiol Ecol 33:35–39

Sjoblad SJ, McClintock JT, Engler R (1992) Toxicological considerations for protein components of biological pesticide products. Regul Toxicol Pharmacol 15:3–9

Tapp H, Stotzky G (1998) Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* in soil. Soil Biol Biochem 30:471–476

USDA (2008) United States Department of Agriculture, Animal and Plant Health Inspection Service, Biotechnology Regulatory Services (2008) General document preparation guidelines for submission to BRS. http://www.aphis.usda.gov/brs/pdf/Doc_Prep_Guidance.pdf. Accessed 4 Jan 2012

USDA (2010) United States Department of Agriculture, Animal and Plant Health Inspection Service, Biotechnology Regulatory Services (2010) User guide for a notification. http://www.aphis.usda.gov/biotechnology/downloads/notification_guidance_0810.pdf. Accessed 4 Jan 2012

US. EPA (1996) Office of chemical safety and pollution prevention harmonized test guidelines, Series 885—Microbial pesticide test guidelines, Group D—Nontarget organism and environmental expression test guidelines, 885.4000—Background for nontarget organism testing of microbial pest control agents. http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPPT-2009-0159-0028. Accessed June 27, 2012

U.S. EPA (2000) Potato leaf roll virus resistance gene (also known as *orf1/orf2*)(006469) Registration eligibility document (BRAD). http://www.epa.gov/oppbppd1/biopesticides/ingredients/tech_docs/tech_006469.htm. Accessed 4 Jan 2012

U.S. EPA (2001) Biopesticide registration action document- *Bacillus thuringiensis* plant-incorporated protectants. http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm. Accessed 4 Jan 12

U.S. EPA (2002) Office of chemical safety and pollution prevention harmonized test guidelines, series 870—Health effects test guidelines, group A—Acute toxicity test guidelines, 870.1100—Acute oral toxicity. http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPPT-2009-0156-0003. Accessed June 27, 2012

U.S. EPA (2004) Plant-incorporated protectant experimental use permit: process and compliance, proceedings. http://www.epa.gov/oppbppd1/biopesticides/pips/pip-eup-workshop/EUP%20Workshop%20main%20Final%20Proceedings.pdf. Accessed 4 Jan 2012

U.S. EPA (2006a) Insect resistance management fact sheet for *Bacillus thuringiensis* (BT) corn products. http://www.epa.gov/oppbppd1/biopesticides/pips/bt_corn_refuge_2006.htm. Accessed 04 Jan 2012

U.S EPA (2006b) Insect resistance management fact sheet for *Bacillus thuringiensis* (BT) cotton products. http://www.epa.gov/oppbppd1/biopesticides/pips/bt_cotton_refuge_2006.htm

U.S. EPA (2007) Guidance on small-scale field-testing and low-level presence in food of Plant Incorporated Protectants (PIPs), pesticide registration notice 2007–2. http://www.epa.gov/PR_Notices/pr2007-2.htm. Accessed June 27, 2012

U.S. EPA (2010a) Biopesticide registration action document – *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production (Vector PV-ZMIR13L) in MON 863 Corn (OECD Unique identifier: MON-ØØ863-5) PC Code: 006484 and *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production (Vector PV-ZMIR39) in MON 88017 Corn (OECD Unique Identifier: MON-88Ø17-3) PC Code: 006498. http://www.epa.gov/pesticides/biopesticides/pips/cry3bb1-brad.pdf. Accessed 04 Jan 2012

U.S. EPA (2010b) Biopesticide registration action document – modified Cry3A protein and the genetic material necessary for its production (Via Elements of pZM26) in event MIR604 Corn SYN-IR604-8. http://www.epa.gov/pesticides/biopesticides/pips/mcry3a-brad.pdf

U.S. EPA (2011a) Tips for Plant-Incorporated Protectant (PIP) Experimental Use Permit (EUP) program submission. http://www.epa.gov/pesticides/biopesticides/pips/pip_hints.htm. Accessed 04 Jan 2012

U.S. EPA (2011b) Current and previously registered section 3 plant incorporated protectants. http://www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm. Accessed 04 Jan 2012

U.S. FDA (1992) United States food and drug administration, guidance to industry on foods derived from new plant varieties. Fed Regist 57:22984–23005, May 29, 1992

Chapter 11
United States Environmental Protection Agency Insect Resistance Management Programs for Plant-Incorporated Protectants and Use of Simulation Modeling

Sharlene R. Matten, Robert J. Frederick, and Alan H. Reynolds

Abstract Widespread adoption of *Bt* crops and persistence of *Bt* toxins expressed in transgenic plants could cause rapid evolution of resistance in pests and lead to the loss of the intrinsic environmental and economic benefits associated with this technology and that of *Bt* microbial pesticides used in organic and conventional agriculture. The United States Environmental Protection Agency (EPA) requires insect resistance management (IRM) programs for plant-incorporated protectants (PIPs) that express insecticidal toxins from the soil bacterium *Bacillus thuringiensis* (*Bt*). The basis for this decision is that maintaining the susceptibility of agricultural pest insects to *Bt* is an important public resource. In contrast to the voluntary pesticide resistance management programs for conventional pesticides, the IRM programs for *Bt* PIPs are mandatory and are unprecedented in their detail, scope, and implementation. EPA has relied on both empirical data and mathematical simulation models to assess the evolution of resistance and evaluate IRM strategies. IRM requirements have changed over the past 15 years in conjunction with the development of new *Bt* PIP products and advances in our understanding of the biological, ecological, genetic, and operational factors that influence the evolution of insect resistance. This chapter focuses on the scientific framework EPA uses to assess and manage the

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risk of insect resistance to *Bt* PIPs with particular interest in the factors that influence resistance and the use of IRM models to evaluate different resistance management strategies.

Keywords Insect resistance management • *Bt* resistance • Refuge strategy • *Bacillus thuringiensis* • Corn • Cotton • Cry toxins • Simulation modeling • Plant-incorporated protectants

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The views expressed in this chapter are those of the authors and do not necessarily represent those of the United States Environmental Protection Agency (EPA). The use of trade, firm, or corporation names in this article is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by EPA of any product or service to the exclusion of others that may be suitable.

11.1 Introduction

Managing resistance of agricultural pests to pesticidal products has been a challenge dating back to the early part of the twentieth century. The development of pesticide resistance in insects, fungi, and weeds is well documented in agriculture (CAST 2004). For example, Whalon et al. (2008) reported 7,747 instances of insect resistance to particular pesticide products. There has been an ongoing debate since the 1950s on how to solve resistance problems and whether there is an over-reliance on crop protection products (Thompson et al. 2008). This debate was heightened in the mid-1980s when the role of regulatory agencies in dealing with pest resistance became more widely discussed (Hawkins 1986). However, until the advent of transgenic crops that express insecticidal proteins isolated from the soil microorganism, *Bacillus thuringiensis (Bt)*[1], in the early 1990s, the prevailing viewpoint was that resistance management was too complex to regulate (Thompson and Head 2001). One reason for this change was the degree of public interest in the evolution of insect resistance to *Bt* crops and to *Bt* microbial pesticides (Glaser and Matten 2003; Thompson et al. 2008).

The rapid and widespread adoption of *Bt* crops in the United States has presented a challenge to their sustainable use. As shown in Fig. 11.1, adoption of *Bt* corn grew from 8% of the corn acreage planted in 1997 to 63% of the corn acreage planted in 2010. Adoption of *Bt* cotton grew from 15% of the cotton acres planted in 1997 to

[1] Transgenic crops expressing *Bt* toxins are also referred to in this document as *Bt* crops or as *Bt* plant incorporated protectants (PIPs). EPA defines a PIP as pesticidal substance that is intended to be produced and used in a living plant, or in the produce thereof, and the genetic material necessary for production of such a pesticidal substance. It also includes any inert ingredient contained in the plant, or produce thereof. [Code of Federal Regulations, Title 40 -Protection of Environment, Part 174.3].

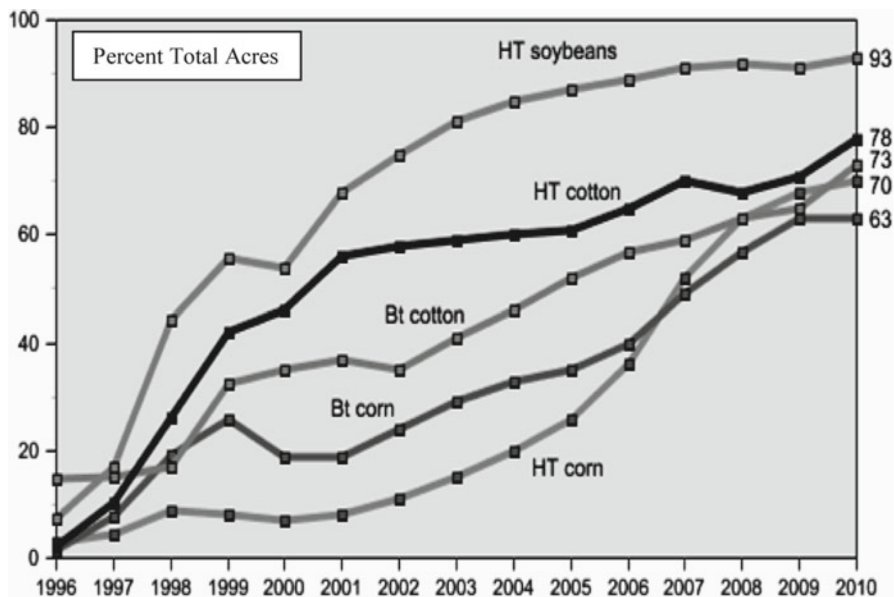

Fig. 11.1 Adoption of *Bt* and herbicide-tolerant (HT) crops in the United States. Data for corn and cotton include varieties with both HT and *Bt* stacked traits. Source: Economic Research Service, USDA

73% of the cotton acres planted in 2010. *Bt* varieties and "stacked varieties," which confer both herbicide tolerance and insect protection, accounted for nearly 80% of all genetically-engineered corn and cotton varieties planted (ERS 2010). Insects exposed to *Bt* PIPs over many generations may evolve resistance at faster rates than they would to conventional pesticides because the insecticidal toxins are expressed at high levels throughout the life of the plant instead of the short period of efficacious levels of conventional pesticides (discussed in USEPA 1998, 2001; Gould 1998; Glaser and Matten 2003). The prolonged high exposure of insects to the toxins in these plants exerts significant selection pressure for resistance development.

While an insect resistance management (IRM) plan is not specifically required under the U.S. pesticide laws or regulations, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) mandates that the EPA ensure that there will be no unreasonable adverse effects from the use of a pesticide when economic factors are taken into account. In 1996, EPA presented the issue of *Bt* resistance and its regulation (and pesticide resistance, more generally) to the Pesticide Program Dialogue Committee (PPDC), an EPA Federal advisory committee. The PPDC recommended that maintaining the susceptibility of insects to *Bt*, whether as a *Bt* microbial pesticide or a *Bt* PIP, was in the "public good" (PPDC 1996). Subsequently, EPA stated that insect resistance to *Bt* PIPs was an unreasonable adverse effect and that IRM programs would be required to maintain the productivity of *Bt* as an important public resource in agricultural production systems (USEPA 1998, 2001; Berwald et al. 2006).

Two important factors were taken into consideration before EPA made the decision to institute IRM requirements for all *Bt* PIPs (USEPA 1998, 2001; Glaser and Matten

2003). First, *Bt* microbial formulations, consisting of spores of one or more different strains of *Bt* and their associated insecticidal crystal proteins, have been used by growers in fruit and vegetable production, especially organic growers, for over 40 years (Walker et al. 2003). The evolution of insect resistance to *Bt* PIPs might affect the efficacy of *Bt* microbial formulations used in organic agriculture (USEPA 1998, 2001; Glaser and Matten 2003). Genes that encode for the production of several of the insecticidal crystal proteins found in *Bt* microbial formulations have also been engineered into *Bt* PIPs. If the target insects developed resistance to the *Bt* toxin(s) expressed in the transgenic plants, then *Bt* microbial pesticides and *Bt* PIPs might also become ineffective, resulting in the loss of *Bt* microbial sprays as a pest control mechanism available to organic agriculture. For example, *Bt* microbial formulations are registered for control of the European corn borer (*Ostrinia nubilalis* Hübner, ECB) in hybrid seed corn production, for control of cabbage looper (*Trichoplusia ni* Hübner) in pepper production, and for control of tomato podworm (*Helicoverpa zea* Boddie) in tomato production (USEPA 2001). *H. zea* is also known as corn earworm (CEW) when it infests corn and as cotton bollworm (CBW) when it infests cotton. Many of the insects targeted by *Bt* microbial formulations are also targeted by *Bt* crops.

Second, the use of *Bt* PIPs has environmental benefits that are worth protecting. Many authors have described important environmental and economic benefits, which have resulted from the use of *Bt* PIP crops, *e.g.*, reduction in exposure to conventional pesticides as a result of lowered conventional pesticide use, reduction in greenhouse gas emissions, mycotoxin reduction in *Bt* PIP corn, and yield increases (USEPA 2001; Glaser and Matten 2003; Brookes and Barfoot 2008; Carpenter et al. 2002; Cattaneo et al. 2006; Fernandez-Cornejo and Caswell 2006; Johnson et al. 2007; Wu 2006). These important benefits would be lost to *Bt* resistance and, inevitably, some growers would choose to return to using less environmentally-friendly conventional pesticides to maintain yield and economic viability.

EPA has required IRM plans for *Bt* PIPs which target major insect pests in corn, cotton, and potatoes (see historical discussion in EPA 2001). *Bt* corn PIPs have been registered for control of stalk boring (lepidopteran) pests such as ECB since 1995 and for control of corn rootworm (coleopteran) pests such as the Western corn rootworm (WCR), *Diabrotica virgifera virgifera* (LeConte), since 2003. *Bt* cotton PIPs have been registered for control of key foliar and boll-feeding pests, including CBW and tobacco budworm (TBW), *Heliothis virescens* L., since 1995.

The evolution of resistance is pest, crop, and *Bt* toxin specific and depends on a number of biological, ecological, genetic, and operational factors. Existing information concerning the biology and ecology of the insect, the genetics of resistance, and spatial/temporal distribution patterns used in the study of insect resistance to chemical pesticides was sufficient to construct reasonable resistance development scenarios that could be used in studying the evolution of insect resistance to *Bt* toxins. Simulation models have proven to be a valuable means of evaluating the influence of different factors on the evolution of resistance in the absence of actual field resistance. Modeling has also been useful in identifying key data gaps. Even at their best, models are just approximations of reality and have associated uncertainties. Therefore, some caution should be exercised when interpreting a model's output.

The science supporting IRM and insect resistance monitoring is complex and is continuing to develop. Maintaining an IRM program requires effective actions of

farmers, seed companies, researchers, and government regulators. IRM programs cannot remain static, but need to be adapted to changes in *Bt* crop technology and in our understanding of the factors that influence the evolution of resistance, to remain sustainable (Glaser and Matten 2003). This chapter focuses on the scientific framework that EPA uses to assess and manage the evolution of insect resistance to *Bt* PIPs, with particular interest in the importance of IRM models and how they are used to evaluate resistance management strategies.

11.2 Public Involvement and Scientific Peer Review

11.2.1 Key Publications and Meetings

IRM requirements for *Bt* crops were developed in consultation with technical experts from industry, academia and government (EPA and USDA) with special consideration of the interests of grower organizations and public interest groups. During all stages of development of IRM plans, technical information was gathered and exchanged at IRM workshops, professional meetings, and through consultations with technical experts (Table 11.1). Especially in the early years, 1995–2001, EPA relied on key reports from expert panels and workshops (Table 11.2), published data, and studies submitted by the registrants to establish a scientific basis for IRM strategies.

11.3 Scientific Peer Review

External scientific peer-review of IRM programs for *Bt* PIPs by the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) has been critical to the development and scientific integrity of the required IRM programs for *Bt* PIPs. The FIFRA SAP is a Federal advisory committee subject to the requirements of the Federal Advisory Committee Act (FACA). The SAP is composed of biologists, statisticians, toxicologists and other experts who provide independent scientific advice to the EPA on a wide-range of health and safety issues related to pesticides. All SAP meetings are open to the public and announced at least 15 days in advance of the meeting. The public is offered an opportunity to provide written and oral comments prior to and during the meeting.

All background documents for each SAP meeting are publicly available in the Office of Pesticide Programs (OPP) regulatory docket and accessible through the federal e-docket portal, http://www.regulations.gov. A SAP report is produced within 90-days following each meeting. From 1992 to 2010, there have been ten SAP meetings related to IRM for *Bt* PIPs (Table 11.3). For example, EPA has held a SAP meeting for most novel *Bt* PIP products (*e.g.*, first corn rootworm-protected *Bt* PIP corn product, 2002) or IRM strategy (*e.g.*, high dose refuge approach, 1998 and 2000; natural refuge, 2004 and 2006; seed blends, 2009 and 2010) (Table 11.3). The SAP reports from 1996 to present are posted electronically on the EPA website, http://www.epa.gov/scipoly/sap/meetings/index.htm.

Table 11.1 Key technical meetings and workshops on *Bt* PIP IRM held with stakeholders between 1995 and 2001

Meetings	Date (month/year)
United States Department of Agriculture (USDA) public forum	April 1996
Pesticide Program Dialogue Committee (an EPA Federal Advisory Committee)	July 1996, January 1999
USEPA public hearings on general IRM issues (public forum)	March and May 1997
USEPA/USDA workshops on IRM for *Bt* corn PIPs and *Bt* cotton PIPs	June 1999 (corn), August 1999 (cotton)
USEPA technical briefing for the *Bt* PIPs reassessment	July 2000
USEPA Workshops on Framework for IRM	2001 (four separate workshops)
USDA regional research committees on European corn borer (NC-205) and on corn rootworm (NCR-46) Annual Meetings	Annually in January
Entomology Society of America (ESA) meetings; Regional meetings	Annually in November/December; Regional meetings in March
Cotton Beltwide Conferences	Annually in January
Meetings with grower groups (*e.g.*, National Corn Growers Association, Illinois Corn Growers Association, Arizona Cotton Growers Association, National Cotton Council)	Variable throughout the year
Meetings with registrants (*e.g.*, Bayer CropSciences, Dow AgroSciences, Monsanto, Pioneer/DuPont, Syngenta Crop Protection)	Regularly
Meetings with the Canadian Food Inspection Agency (CFIA) to harmonize IRM requirements in Canada and the U.S. for *Bt* PIP corn and for *Bt* PIP potato	Sporadic

Table 11.2 Key publications that influenced the development of the IRM program elements: 1995 to 2001

Key publications	Reference
USDA NC-205: ECB ecology and management	Mason et al. (1996)
USDA NC-205:*Bt* corn IRM	Ostlie et al. (1997), Supplement in October1998
USEPA White Paper on IRM for Bt Crops	USEPA (1998)
Union of Concerned Scientists “Now or Never” Report	Mellon and Rissler (1998)
International Life Sciences Institute Report (ILSI) “An Evaluation of Insect Resistance Management in *Bt* Field Corn”	ILSI (1999)
USEPA/USDA Position Paper on IRM for *Bt* Crops	USEPA and USDA (1999)
USEPA Response to the Greenpeace et al. Petition	USEPA (2000)
USDA Agriculture Research Service *Bt* Cotton IRM Report, “*Bt* cotton and management of the tobacco budworm-bollworm complex”	Hardee et al. (2001)
USEPA *Bt* Crops Reassessment Document	USEPA (2001)

Table 11.3 List of FIFRA SAP meetings related to scientific issues associated with IRM for *Bt* PIPs

FIFRA SAP meetings	Date(s)	Topics covered
Subpanel on Plant Pesticides[a]	December 18, 1992	Defining risks of PIPs
Subpanel on Plant Pesticides	March 1, 1995	Monsanto Company's application for registration of *Bt* subspecies *tenebrionis delta* endotoxin
Subpanel on *Bt* Plant-Pesticides and Resistance Management	February 9–11, 1998	*Bt* PIP insect resistance management
Issues Pertaining to the *Bt* Plant-Pesticides Risk and Benefit Assessments	October 18–20, 2000	Human health and environmental risk assessment and insect resistance management of PIPs
Corn Rootworm Plant-Incorporated Protectant Insect Resistance Management and Nontarget Insect Issues	August 27–29, 2002	Nontarget insect issues, insect resistance management issues
Product Characterization, Human Health Risk, Ecological Risk, And Insect Resistance Management For *Bt* Cotton Products	June 8–10, 2004	Widestrike® cotton human health/ecological risk and insect resistance management; Bollgard® and Bollgard® II insect resistance management
Refuge of Non-Cotton Hosts for Monsanto's Bollgard® II Cotton	June 13–15, 2006	Sampling and methodology, statistical analyses, effective refuge calculation and modeling, data/results interpretation
Evaluation of the Resistance Risks from Using 100% Bollgard® and Bollgard® II Cotton as Part of a Pink Bollworm Eradication Program in the State of AZ	October 24–25, 2006	Simulation modeling and pink bollworm eradication
Resistance Risks from Using a Seed Mix Refuge with Pioneer's Optimum AcreMax1® Corn Rootworm-Protected Corn	February 23–25, 2009	IRM issues associated with seed blend strategy for Optimum AcreMax 1 Corn Rootworm-Protected Corn
Scientific Issues Associated with IRM for SmartStax™ Refuge-in-the-Bag, a *Bt* corn PIP	December 8–9, 2010	IRM issues associated with seed blend strategy for SmartStax™ Refuge-in-the-Bag

[a]Plant-pesticides were renamed plant-incorporated protectants (PIPs) in 2001 following publication of PIP regulations in the Federal Register (Regulations Under the Federal Insecticide, Fungicide, and Rodenticide Act for Plant-Incorporated Protectants (Formerly Plant- Pesticides) Federal Register 66(139): 37771–37817)

11.4 Data Considerations

Under FIFRA, all pesticide products must be registered by the EPA prior to their manufacture, distribution, and use in the United States. EPA has been developing and requesting IRM data in support of registrations of *Bt* PIPs since 1995. Throughout this process, EPA sought regular scientific advice from the SAP (noted above in Sect. 11.3) on factors that influence the evolution of *Bt* PIP resistance and strategies that may delay insect resistance to *Bt* PIPs for ECB and CRW and cotton pests such as TBW, CBW, and pink bollworm (*Gossypiella pectinophora* Saunders, PBW).

EPA developed eight data elements used to develop IRM plans consistent with the scientific advice of the SAP from 1992 to 2010 (see Table 11.3). Elements (1)–(4) displayed in Table 11.4 are the data used to evaluate the likelihood of resistance evolution and relative durability of the proposed IRM strategy. Elements (5)–(8) are post-registration (*e.g.*, stewardship activities) measures for resistance monitoring, remedial action, grower education, and compliance assurance. During the risk assessment phase of the registration process, these plans are evaluated for technical adequacy. Plans may be modified based on information provided in the annual reports and discussed with the registrants.

To address Elements (1)–(4), the registrant provides information from published literature, specific data from laboratory and field, and simulation modeling to evaluate the risk of resistance evolution for the target pests, and the rationale to support the proposed IRM strategies to delay resistance. Figure 11.2 illustrates the resistance assessment and management decision scheme. In this scheme, the risk assessor evaluates the risk of resistance evolution for each *Bt* PIP as expressed in a plant. Each assess-

Table 11.4 Data used to assess and manage insect resistance to *Bt* PIPs

Data	EPA assessment	Annual reporting
Pre-registration measures		
(1) Target organism(s) biology and ecology (2) Target organism(s) susceptibility to toxin(s), *e.g*. "dose," mortality (3) Mode of action, genetics of resistance, potential for cross resistance (4) Crop and target insect specific IRM simulation models	Data used to assess risks of resistance and development/ implementation of IRM field requirements, *e.g.*, structured refuge.	N/A
Post-registration measures: education, monitoring, and mitigation		
(5) Resistance monitoring plan	Resistance monitoring program	Yes
(6) Remedial action plan	When triggered, implement remedial action for "suspected" and "confirmed" resistance	As needed included in resistance monitoring report
(7) Compliance assurance plan	Compliance assurance program	Yes
(8) Grower education plan	Grower education program	Yes

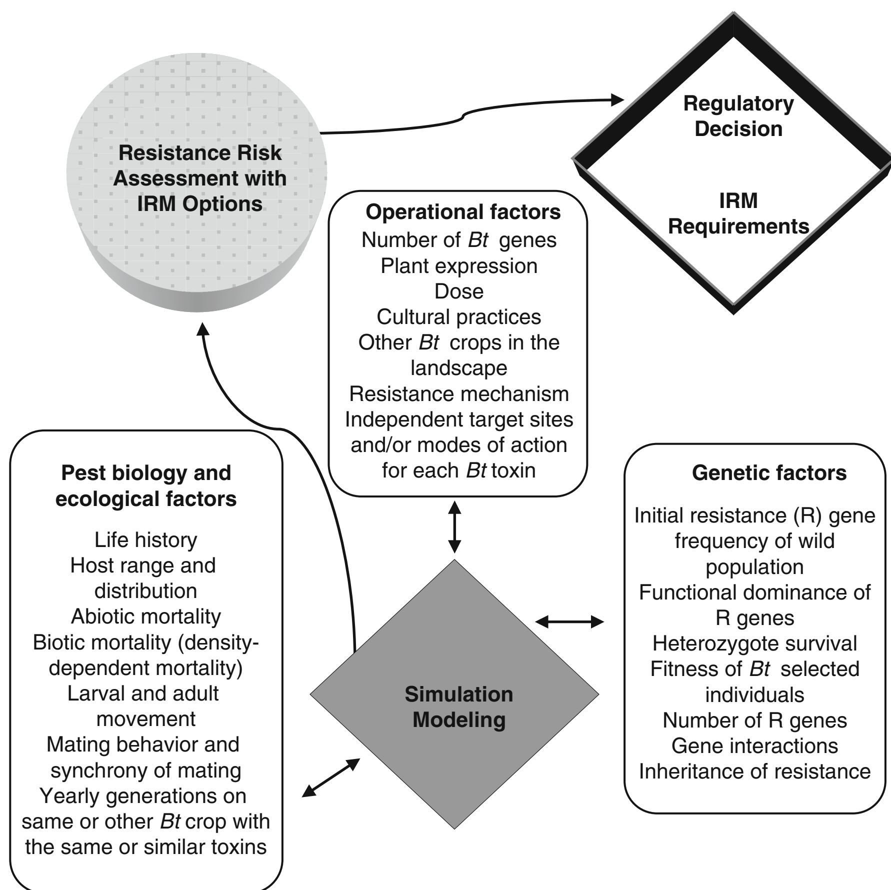

Fig. 11.2 Insect Resistance Assessment and Management Decision Scheme

ment involves a thorough examination of three sets of resistance factors that influence the evolution of pest resistance to *Bt* PIPs.

1. Pest biology and ecology factors, *e.g.*, biotic mortality, abiotic mortality, pest movement (larval and adult), mating behavior and synchrony of mating, yearly generations on same or other *Bt* crop with the same or similar toxins, and host range of the pest;
2. Genetic factors of the pest, *e.g.*, initial resistance (R) gene frequency of wild population, functional dominance of R genes, fitness of *Bt*-selected individuals, and number of R genes involved in resistance; and
3. Operational factors, *e.g.*, number of *Bt* genes deployed, expression of *Bt* toxin(s), dose of *Bt* toxin(s), mode of action, resistance mechanism, cross-resistance, use of insecticides, and cultural practices.

As shown in Fig. 11.2, simulation modeling is used as a tool to integrate all of the resistance factors to predict the evolution of resistance and compare resistance management options. For example, these models allow for a qualitative comparison of different refuge sizes and deployment patterns. The risk assessor characterizes the uncertainties in the assessment of the evolution of resistance and modeling. The assessment is refined as the understanding of the resistance factors affecting the evolution of *Bt* PIP resistance becomes clearer. The risk assessor provides the risk manager with IRM recommendations based on the current understanding of the likelihood of the evolution of resistance. The risk manager makes the regulatory decision to establish the IRM requirements. The risk manager considers the scientific recommendations from the risk assessor, but also other factors such as grower costs and compliance, resistance monitoring, and remedial action if resistance should occur.

11.5 High Dose Structured Refuge Strategy

When compared to chemical insecticides, *Bt* PIPs expressed in crop plants are unique in their ability to produce continuous, season-long, high doses of the insecticidal toxin. Although many resistance management strategies have been proposed to delay pest resistance to *Bt* PIP crops (*e.g.*, Gould 1998; Roush 1997; Tabashnik 1994a; Andow 2002; Zhao et al. 2003; Bates et al. 2005), the high dose refuge strategy has been the one most widely used. The high dose refuge strategy is based on evolutionary theory described by Comins (1977), Georghiou and Taylor (1977) and others (Taylor 1983; Tabashnik 1986). The high dose refuge strategy has been tested empirically in small-scale greenhouse experiments with *Plutella xylostella* L. (diamondback moth, DBM) (Liu and Tabashnik 1997; Shelton et al. 2000; Zhao et al. 2003, 2005; Tang et al. 2001). The high dose refuge strategy includes both sufficiently high concentrations of toxin in the plants to kill most heterozygous (RS) insects and the use of refuges to provide susceptible (SS) insects. EPA adopted the high dose refuge strategy to manage insect resistance to *Bt* PIP crops given the recommendations of the FIFRA SAP (1995, 1998, 2000) and general agreement among stakeholders (see discussion in EPA 2001, Andersen and Matten 2002; Glaser and Matten 2003; Matten and Reynolds 2003; Matten et al. 2004). The high dose refuge strategy has three key assumptions.

1. Resistance will be recessive and conferred by a single locus with two alleles resulting in three genotypes: susceptible homozygotes (SS), heterozygotes (RS), and resistant homozygotes (RR).
2. Resistance alleles will be present at a low frequency in the environment.
3. There will be random or preferred mating between resistant and susceptible adults.

A structured refuge is a non-*Bt* portion of a grower's field or set of fields that provides for the production of SS insects that may randomly mate with rare RR insects emerging in *Bt* PIP fields to produce susceptible RS heterozygotes that will be killed by the *Bt* PIP crop. In combination, the high dose and use of a refuge will reduce the number of resistance alleles in target pest populations and effectively delay the evolution of resistance.

A key question is how to estimate the number of susceptible adults needed in the refuge. In 1998, EPA adopted the SAP's recommendation that a refuge should produce at least 500 susceptible adults for every resistant adult in the transgenic crop area (SAP 1998). The SAP noted that the structured refuge should be planted at the same time as the *Bt* PIP crop to provide susceptible insects in synchrony with the emergence of putative resistant insects.

Although the high dose refuge strategy has been the preferred IRM strategy to manage the evolution of resistance to *Bt* PIPs, effective IRM is still possible even if the transformed plant does not express the *Bt* PIP(s) at a high dose to control target pests, *e.g.*, increase the size of the refuge. The concern with the lack of a high dose is that partially resistant heterozygous (RS) insects will survive; thus, increasing the frequency of R alleles in the population. For this reason, numerous IRM researchers and expert groups have stated that non-high dose *Bt* expression presents a greater resistance risk relative to high dose expression (Roush 1994; Gould 1998; Onstad and Gould 1998a, b; SAP 1998; ILSI 1999; Mellon and Rissler 1998; SAP 2001). The implications of dose on selection for resistance are discussed in more detail in Sect. 11.9.

11.6 Factors That Influence Resistance

An important objective of an IRM strategy is to find ways to reduce the selection pressure and the fitness advantage of resistance alleles. Understanding the biological, genetic, and operational factors that affect the evolution of resistance (see Fig. 11.2) is crucial to developing an effective IRM strategy. The EPA asks each registrant to provide data that will address the biological, genetic, and operational factors that affect the evolution of resistance to the *Bt* PIPs expressed in the crop plant.

11.7 Pest Biology and Ecological Factors

As an IRM strategy is developed and implemented, each pest's unique biology and ecology must be factored into the plan. For example, how far the larvae move within the field and how far the adults move affects the distance between the refuge and the *Bt* PIP crop. The susceptible insects from the non-*Bt* refuge need

to be in close enough proximity to mate with the resistant insects that emerge from the *Bt* fields to produce heterozygous offspring that are fully susceptible to the *Bt* toxin. In addition, to understanding larval and adult movement, other important biological and ecological factors considered are: number of insect generations produced each year, mating behavior and oviposition behavior, host range of the insect and host utilization, fecundity, overwintering behavior and survival, and population dynamics (see Fig. 11.2). Several key factors are discussed below.

11.7.1 Larval Movement

Larval movement may be a concern because heterozygotes that are at least partially resistant may begin feeding on *Bt* plants then move to nearby non-*Bt* plants in order to complete their development; thus, possibly defeating the high-dose strategy and increasing the risk of resistance (Ives et al. 2010; Glaum et al. 2012; SAP 2011). Larval movement differs between insect species. In many species, larvae have been shown in the laboratory and the field to preferentially move from *Bt* plants to non-*Bt* plants (Parker and Luttrell 1999; Tang et al. 2001; Hibbard et al. 2003, 2004, 2005) or from the upper parts of plants, where the toxin levels are higher, to lower parts of the plants, where the toxin levels are lower (Gore et al. 2002). In contrast, PBW have been shown to have no feeding preference between non-*Bt* and *Bt* cotton for PBW (Heuberger et al. 2008). Modeling was used to study the effects of PBW larval feeding behavior, *Bt* gene flow among plants, refuge size, and dominance of resistance on the evolution of resistance (Heuberger et al. 2011). In situations of moderate or high *Bt* gene flow among plants and resistance was intermediately dominant, resistance evolution was accelerated in some scenarios when non-*Bt* cotton refuges were 5 or 20% of the cotton acreage. Larval movement and indiscriminant feeding among *Bt* and non-*Bt* cotton plants further increased the rate of evolution of resistance. Simulation modeling has been used to evaluate the effect of larval movement on the rate of resistance evolution in fields planted with seed mixtures of non-*Bt* and *Bt* plants (Davis and Onstad 2000; Onstad and Gould 1998a; Mallet and Porter 1992; Peck et al. 1999; Ives et al. 2010). On at least two occasions, the SAP advised EPA of the negative effects of ECB (and SWCB) larval movement on the durability of seed mixtures as an IRM strategy (SAP 1998, 2011).

11.7.2 Dispersal

The mobility of pests is influenced by the properties of a given population as well as a variety of biotic and abiotic factors (Kennedy and Storer 2000). The dispersal patterns of insects between the refuge and the *Bt* PIP fields will impact the evolution of resistance. For example, Andow and Ives (2002) modeled the effect of reducing

dispersal by ECB females in the refuge fields. Results from this model indicated that reduced female dispersal may slow the rate of evolution of resistance under high dose conditions. Susceptible females will be more likely to lay eggs in the separate refuge fields than in the *Bt* PIP fields and escape exposure to the *Bt* PIP, which will reduce the selection pressure for resistance.

Barriers or deterrents to dispersal of *Bt* susceptible insects from the refuge will decrease the opportunity for random mating and result in assortative mating patterns. Caprio et al. (2004) looked at dispersal using a source-sink analogy. Source fields (non-toxic plants) are those where there is net population growth and insects will emigrate. Sink fields (toxic plants) are those where the insect deaths are greater than births and there is net immigration.

Microhabitat factors including relative humidity and plant density (DeRozari et al. 1977; Hellmich et al. 1998) affect dispersal. Hunt et al. (2001) showed that there was a microclimate preference by ECB for irrigated corn fields and this might limit dispersal into non-*Bt* corn fields. These results suggest that nonrandom mating, which may compromise IRM plans, may occur more often in irrigated areas if refuge is not placedin close proximity or within the fields.

Showers et al. (2001) indicated that males and females dispersed great distances, 23–49 km, in just a few nights and that some disperse 14 km, although most males were recaptured at a distance of 800 m from the release point. These data support the placement of the non-*Bt* refuge within a half-mile of the *Bt* PIP corn fields. In practical terms, resistant moths should encounter an ample supply of susceptible moths whether they mate in an aggregation site near their field of origin or move to a more distant aggregation site to mate. Qureshi et al. (2005) demonstrated that more than 90% of marked ECB adults were recaptured within 300 m of the release point, a large 50 ha center pivot in irrigated *Bt* PIP corn fields. However, large numbers of feral adults and virgin females (marked and feral) were captured throughout the study fields indicating that there is dispersal of ECB from the non-*Bt* (refuge) corn fields into the *Bt* PIP corn fields that allows some genetic mixing of the two populations. Qureshi et al. (2006) found the same pattern of dispersal for SWCB, *i.e.*, 90% of marked SWCB adults were recaptured within 300 m of the release point, large numbers of feral adults and virgin fields were captured throughout the study fields. Qureshi et al. (2005, 2006) indicated that there were refuge sources of feral adults approximately 587–1,387 m from the edge of the fields. Dalecky et al. (2006) demonstrated that predispersal mating did occur in ECB, but once present locally, immigrant individuals had the same probability of mating with any locally present individual of the other sex.

11.7.3 Oviposition

Bt crops may affect oviposition behavior and egg production. Insecticidal toxins produced by the plant could provide a selective advantage to less susceptible heterozygotes or homozygous resistant insects. Therefore, it would be advantageous

to have refuges of plants without the *Bt* toxin available for oviposition and larval development to ensure continuation of the homozygous-susceptible (SS) insects and maintenance of the susceptible allele in the population. For example, TBW and CBW had different oviposition patterns, with the former, the number of eggs increased as the size of imbedded refuges increased, while there was no measurable change in egg numbers relative to refuge size (Caprio et al. 2004). However, Torres and Ruberson (2006) did not find any effect on the temporal or spatial patterns of oviposition for TBW and CBW in *Bt* PIP cotton growing areas. Similarly, no significant differences in egg densities were found for *H. armigera* (sometimes referred to as old world bollworm) on Cry1Ac cotton and non-transgenic varieties in China (Wu et al. 2003). Hibbard et al. (2004) reported density independent deposition of WCR eggs on corn plants. Jackson et al. (2003) found no differences in CBW oviposition behavior on conventional (non-transgenic) cotton, Bollgard® cotton (Cry1Ac), or Bollgard® II cotton (Cry1Ac and Cry2Ab) in the field.[2] The FIFRA SAP commented that second generation ECB females will discriminate against ovipositing on damaged non-*Bt* (refuge) plants versus undamaged *Bt* plants grown near each other as is the case for seed mixtures (SAP 2011). As a result more eggs will be laid on the undamaged *Bt* plants, reducing the effectiveness of the seed mixture to delay resistance evolution.

11.7.4 Diapause

Diapause (a physiological means of dormancy) in a insect is a mechanism to survive unfavorable environmental conditions such as temperature extremes, drought, reduced food availability. When larvae begin diapause and when they emerge from diapause may be affected by developmental vigor and genetics. An overwintering fitness cost associated with the resistance allele may result in lower emergence rates such as seen in laboratory studies of resistant-PBW on non-Cry1Ac cotton plants (Carrière et al. 2001b, c, d).

11.7.5 Population Dynamics

Population size and stability are influenced by a complex variety of biological and environmental factors that impact upon reproduction and growth (Kennedy and Storer 2000). For modeling purposes, factors affecting the population dynamics include density-dependent mortality, overwintering success, and environmental impacts. Density-dependent survival is observed when eggs and/or larvae have lower survival at higher densities when, for example, predation, parasitism, or competition for food increases as insect density increases. Research studies have focused

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[2] Bollgard® cotton and Bollgard II® cotton are registered products of Monsanto Company.

on the effect of density-dependent mortality on the evolution of CRW resistance to *Bt* crops (Onstad et al. 2001, 2003; Onstad 2006; Crowder et al. 2005a, b; Storer et al. 2006; Hibbard et al. 2010; SAP 2011). The challenge is to measure density-dependent survival accurately and precisely in the field when there are many factors which reduce mortality in the field, such as temperature, humidity, and soil conditions. The SAP noted that a density-dependent adjustment factor may provide a false sense of accuracy if the original estimates of mortality are not precise or repeatable so that dose mortality can be estimated accurately (SAP 2011).

11.7.6 Alternate Hosts

Assessing the insect's host range can provide an indication of whether alternate hosts will be able to serve as a refuge for resistance adults emerging from *Bt* PIP crops fields. Host utilization and population dynamics are key factors in determining whether alternate hosts will be a suitable refuge. An alternative host would need to provide sufficient numbers of *Bt* susceptible adults to dilute any resistance genes arising from *Bt* PIP crop fields. Key factors in this analysis are: host range, distribution of alternate hosts (source potential for production of susceptible insects), percentage of population on each host, survivorship and fecundity on alternate hosts, phenology of the host; fitness costs of insects on alternate hosts, behavior and life history on alternate hosts. The extent to which an insect utilizes plant hosts depends on a number of factors, including movement and dispersal of insects on alternate hosts, agricultural/cropping practices (*e.g.*, landscape mosaics), land use patterns, natural plant fauna, climate, and seasonal variability (*e.g.*, weather, changing cropping patterns, etc.)

11.8 Genetic Factors

11.8.1 Resistance Allele Frequency

Resistance allele frequency is a primary concern in the evaluation of resistance evolution. Spatially-explicit, stochastic modeling by Peck et al. (1999) indicated that the effect of initial gene frequency depended on the size of the region explored (scale of the model). In general, the higher the initial resistance allele frequency in a population, the more rapid the frequency of resistance alleles will increase.

11.8.2 Degree of Dominance of R Alleles

The degree of dominance of resistant alleles affects the rate of resistance in a population. If the alleles conferring resistance in a population are rare, then they

would occur mostly in heterozygotes; resistant homozygotes would be even rarer and the evolution of resistance would be relatively slow. Resistance to *Bt* PIPs expressed at a high dose in the plant is assumed to be conferred by rare, recessive alleles. Heterozygotes with a recessive allele will be phenotypically susceptible to the *Bt* PIP. Heterozygotes with a fully dominant resistance allele will show the same survival characteristics as homozygous resistant insects.

Inheritance of resistance can vary from completely dominant to completely recessive as illustrated by the following studies. Bioassay results from TBW, PBW, and DBM show that the dominance of their resistance to *Bt* toxins decreases as toxin concentration increases (Tabashnik and Carrière 2007). At low toxin concentrations, survival is relatively high for all genotypes. However, at high toxin concentrations, survival of RS and SS insects is relatively low compared to RR insects. In general, inheritance of resistance can vary from completely dominant to completely recessive as the concentration of *Bt* toxin is increased from low to high. When dominance is physiologically determined, heterozygote survival will not depend on toxin concentration. Simulation modeling has been used to examine the sensitivity of this parameter on evolution of resistance (see Bourguet et al. 2000).

Gould et al. (1992, 1995) reported that resistance was nearly completely recessive in a laboratory- selected TBW resistant strain, which had a single major gene conferring 10,000-fold resistance to Cry1Ac. Tabashnik et al. (1997b) characterized three DBM-resistant strains, two of which (NO-QA and PEN) had recessive R alleles and one (PH1) had a dominant R allele. Sayyed et al. (2003, 2005) reported multiple resistance mechanisms in resistant field populations of DBM. Resistance was determined to be incompletely recessive in resistant male crosses, but was incompletely dominant in resistant female crosses, indicating possible maternal influence on resistance expression. Akhurst et al. (2003) reported that a resistant *H. armigera* strain was incompletely recessive for resistance and there were fitness costs associated with resistance. Resistance was recessive when the resistant strain was tested on 4-week-old cotton (Bird and Akhurst 2004), but was partially dominant when tested on 15-week-old cotton with 75% lower Cry1Ac concentrations in the leaves (Bird and Akhurst 2005). In other *H. armigera* strains, resistance was semi-dominant (Gunning et al. 2005; Xu et al. 2005; Kranthi et al. 2006).

Work conducted at the University of Arizona identified three mutant resistance alleles (*r1, r2,* and *r3*) of a cadherin gene (*BtR*) are tightly linked with recessive resistance of PBW to Cry1Ac (Morin et al. 2003; Tabashnik et al. 2004, 2005b). Each *r* allele has a deletion predicted to eliminate at least eight amino acids upstream of the putative Cry1Ac-binding region of the cadherin protein (Morin et al. 2003). Each *r* allele conferred different sensitivities to the Cry1Ac toxin (Morin et al. 2003; Carriére et al. 2006;). Mutations in the cadherin gene have also been shown to have tightly linked with recessive resistance to Cry1Ac in TBW, another lepidopteran pest of cotton (Gahan et al. 2001).

11.8.3 Reproductive Fitness and Fitness Costs

Changes to the genetic makeup of an insect (*e.g.*, the presence of resistance alleles) may impose a fitness cost. This is demonstrated as a decreased fitness of a heterozygote (resistant) relative to the fitness of fully susceptible individuals when grown on non-*Bt* plants. A review of the literature indicates that fitness costs are frequently associated with resistance to *Bt* toxins (Ferré and Van Rie 2002; Groeters et al. 1994). One example is the decreased fitness of Cry1Ac-resistant PBW studied by researchers from the University of Arizona (Tabashnik et al. 2003a). Carrière et al. (2004a, 2005) were able to determine that Cry1Ac resistance was linked to overwintering fitness costs. These authors indicated that fitness costs of resistance are known to be affected by genotype-environment interactions. Researchers suggest that manipulation of fitness costs can enhance the success of the refuge strategy (Carrière et al. 2001b, c, d, 2004a, 2005; Gassmann et al. 2009).

11.8.4 Cross-Resistance

An often asked question is why is resistance a concern if there are different *Bt* PIPs. The answer is the potential for cross-resistance in which resistance to one *Bt* toxin confers resistance to another *Bt* toxin. Cross-resistance is a risk to the continued efficacy of all *Bt* PIPs; however, it is of special concern for pyramided *Bt* PIPs in which multiple *Bt* PIPs toxins are expressed simultaneously in the same plant to control the same set of insect pests. Cross-resistance would neutralize the benefits of "redundant" "killing" achieved through the expression of two of more *Bt* toxins with different modes of action. The literature is abundant with examples of how selection for resistance to one toxin can result in cross-resistance to other toxins (*e.g.*, Ferré et al. 2008; Gould et al. 1992; McGaughey and Johnson 1992; Moar et al. 1995; Tabashnik et al. 1996).

Cross-resistance is common among *Bt* toxins in the Cry1A family, and/or Cry1Fa and Cry1J toxins, but less common between other Cry toxins (Ferré et al. 2008). Competition binding studies conducted with brush border membrane vesicles (BBMV) isolated from a number of insect species indicate that Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja share membrane receptors, but there are unique membrane receptors for each toxin. Binding studies conducted with ECB BBMV showed that Cry1Ab and Cry1Ac recognized the same membrane receptor, but with different binding affinities, while Cry1B recognized a different receptor (Denolf et al. 1993) and Cry1Fa and Cry1Ab (or Cry1Ac) had limited shared binding (Hua et al. 2001). A Cry1Fa resistant line of ECB (>3,000-fold) conferred limited cross-resistance to Cry1Ac (6.9-fold). Binding studies conducted using TBW BBMV indicated that Cry1Fa and Cry1Ja share the Cry1Aa binding site, but each protein also has unique binding sites (Jurat-Fuentes and Adang 2001). These authors proposed a receptor-binding scheme for TBW that shows Cry1Fa, Cry1Ab,

and Cry1Ac binding to the Cry1Aa binding site and each protein also binding to unique binding sites**.** According to this scheme, an altered Cry1Aa binding site would likely result in TBW resistance to all four Cry toxins, but the unique binding sites also play a role in toxicity. In later experiments, Jurat-Fuentes and Adang (2006) demonstrated that a cadherin-like protein, HevCaLP, is the functional receptor for Cry1Ac binding in a highly-resistant (>300,000-fold) TBW colony (YHD2), although it is not a receptor for Cry1Fa (130-fold resistant). These results suggest that the Cry1Fa and Cry1Ac share a binding site which is not a cadherin-like protein and that cross-resistance would be due to modification of some other receptor. Like TBW, binding experiments using BBMV isolated from midgets of *H. armigera and Spodoptera exigua* (beet armyworm) indicated that Cry1Fa, Cry1Ja, and Cry1Ac share a common membrane receptor (Hernández and Ferré 2005).

Collectively, these studies suggest that Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja protein binding to a common site is perhaps the biochemical basis of multiple resistance and cross-resistance among these five proteins in several different insect species (Hernández and Ferré 2005). Such studies stress the importance of establishing a binding site model for each species to develop an appropriate resistance management strategy.

The probability of cross-resistance is typically examined on three levels: (1) structural similarity between the proteins, which is indicative of mode of action; (2) characterization of elements of the mode of action, such as the biophysical nature of binding of the *Bt* toxins to the target insect midgut; and (3) demonstration (*e.g.*, resistant colony work) that the individual *Bt* toxins are effective in controlling resistance to the another *Bt* toxin. Analyses of resistance to *Bt* toxins indicate that cross-resistance occurs most often with proteins that are similar in structure (Tabashnik 1994a; Gould et al. 1995). For example, lack of sequence similarity would suggest that cross-resistance between *Bt* toxins would be unlikely. Binding studies using insect midgut brush border membrane vesicles (BBMVs) or purified receptors in blot-type assays provide information on the binding patterns of *Bt* toxins and potential for cross-resistance. For example, a TBW strain (YHD2) selected for Cry1Ac resistance developed 10,000-fold resistance to this toxin and was cross-resistant to Cry1Aa, Cry1Ab, and Cry1Fa toxins, but not to Cry2A toxins (Gould et al. 1995). Other TBW strains developed about 200-fold resistance to Cry1Ac and cross-resistance to Cry2Aa1 (Gould et al. 1992).

Insect resistant colonies have been the most direct method to examine the potential for cross-resistance. For example, ECB selected for resistance against Cry1Ab did not confer resistance to Cry1F (Pereira et al. 2010; Siqueira et al. 2004). These results suggest that there is a lower probability of cross-resistance between Cry1Ab and Cry1F toxins. In other studies, three Cry1Ab-resistant ECB colonies, along with two ECB susceptible colonies, were assayed for their response to purified Cry1Ab, Cry1Ac, Cry2Ab2, and a version of the Cry1A.105 protein (see discussion in USEPA 2010e). Results of the bioassays indicated that all three Cry1Ab-resistant colonies were resistant to Cry1Ab and Cry1Ac, but remained susceptible to Cry1A.105 and Cry2Ab2, which indicated that resistance to Cry1Ab did not confer cross-resistance to Cry1A.105 and Cry2Ab2 (USEPA 2010e). PBW selected for Cry1Ac resistance (AZP-R) resulted in 200-fold resistance in this colony, high

levels of cross-resistance for Cry1Aa and Cry1Ab, low levels for Cry1Bb, and no-cross-resistance for Cry1Ca, Cry1Da, Cry1Fa, Cry1Ja, Cry2Aa, and Cry9Ca toxins (Tabashnik et al. 2000b). A study conducted with Cry1Ac-resistant PBW and Cry2Ab-resistant PBW resulted in asymmetrical cross resistance to Cry2Ab. In this case, selection for Cry2Ab resistance in PBW resulted in 420-fold increase in Cry1Ac resistance, while selection for Cry1Ac resistance did not result in concomitant Cry2Ab resistance (Tabashnik et al. 2009a). In Australia, Caccia et al. (2010) demonstrated that alteration of target binding sites is the most likely means that field populations evolve resistance to Cry2A proteins in *Helicoverpa* spp.

A highly resistant TBW colony (YHD2) to Cry1Ac (over 10,000-fold) was highly cross-resistant to Cry1Ab and Cry1Fa, but only moderately cross-resistant to Cry2Aa, and almost nonresistant to Cry1Ca and Cry1Ba (Gould et al. 1995). Selection of another colony of TBW (CP73-3) with Cry1Ac resulted in broad, but lower levels of resistance to Cry1Ac (50-fold), Cry1Ab (13-fold), and Cry2A (53-fold), as well as Cry1Aa, Cry1Ba (Gould et al. 1992). While *Bt*-resistant insect colonies are imperfect tools for predicting what will happen in the field, they are the best tools available for looking at potential resistance mechanisms.

11.9 Operational Factors

11.9.1 Toxin Susceptibility and Dose

The susceptibility of insects to *Bt* toxins is a major factor in the consideration of resistance management strategies. Most important is the survival of the heterozygotes. Heterozygotes carrying a resistance allele are less susceptible to *Bt* toxins than homozygous susceptible individuals (Carrière et al. 2004b). Therefore, it is possible to have toxin levels in plants that are sublethal for the former while lethal to the latter. Proportionately more heterozygotes will survive and result in assortative mating, increasing the probability of homozygous-resistant insects. Toxin levels may change during the growing season so that dominance of resistance to Bt crops can vary as the concentration of *Bt* toxin changes. This was demonstrated by Bird and Akhurst (2004, 2005) in *H. armigera*. Mahon and Olsen (2009) fed *H. armigera*-resistant (RR, RS) and fully-susceptible to Cry2Ab on cotton containing Cry1Ac and Cry2Ab toxins and found that survival of all three genotypes was limited, but increased as the level of Cry1Ac decreased during the growing season.

In practice, a *Bt* PIP could be considered to provide a high dose if verified by at least two of the following five approaches (SAP 1998, 2001): (1) serial dilutions bioassay with artificial diet containing lyophilized tissues of *Bt* plants with tissues from non-*Bt* plants as controls; (2) bioassays using plant lines with expression levels approximately 25-fold lower than the commercial cultivar determined by quantitative enzyme linked immunosorbent assay (ELISA) or some more reliable technique; (3) surveys of large numbers of commercial plants in the field to make sure that the cultivar is at the lethal dose (LD) 99.9 or higher to ensure that 95% of heterozygotes would be killed (Andow and Hutchison 1998); (4) similar to approach 3, controlled

infestation with a laboratory strain of the pest that had an LD50 value similar to field strains; and (5) determining whether a later instar of the targeted pest could be found with an LD50 that was approximately 25-fold higher than that of the neonate larvae; if so, the later stage could be tested on the Bt crop plants to determine whether ≥95% of the larvae were killed.

The methods described above were focused on foliar-feeding lepidopteran pests, such as TBW, CBW, and ECB. CRW feeding behavior and survival and root expression data can be used to estimate the dose of CRW-protected Bt PIP corn. Simulation modeling is used, in part, to compare the relative rate of evolution of resistance for *Bt* PIPs at different doses, not just a high dose. Modeling indicates that *Bt* PIPs expressed at a non-high dose will lead to greater survival of RS insects and increase the rate of the evolution of resistance as compared to a high dose expression level (*e.g.,* Roush 1994).

When registering a *Bt* PIP, the registrant must evaluate dose for each of the major target pest as part of their registration package to support a proposed IRM strategy. If there are multiple toxins then the dose of each toxin may be evaluated separately and/or collectively. For example, a single *Bt* PIP plant may express a high dose of the *Bt* toxin to control one target pest, but express a non-high dose of the *Bt* toxin to control (or suppress) another target pest. If there is pyramiding of multiple *Bt* genes in a single plant then the assessment of dose becomes more complicated. For example, a *Bt* PIP plant pyramided with two *Bt* genes might express one *Bt* toxin at either a high dose or non-high dose for control of an insect and the second *Bt* toxin at a either a high dose or non-high dose for control of the same insect. This means that there are four possible dose outcomes for a two *Bt* toxin PIP plant. This process is repeated for each target pest. The dose outcomes for each pest need to be considered in the development of IRM strategies for *Bt* PIPs.

11.9.2 Plant Expression

While seemingly straightforward, determining the toxin levels in plant tissue is not a simple matter. The toxin levels in plants are known to be highly variable depending on the cultivar (Adamczyk and Sumerford 2001; Adamczyk and Gore 2004; Adamczyk and Meredith 2004; Gujar et al. 2004, 2007), between plant parts (Nguyen et al. 2007; Olsen et al. 2005; Wan et al. 2005; Abel and Adamczyk 2004; Adamczyk and Sumerford 2001; Gore et al. 2001, 2002; Greenplate 1999; Olsen and Daly 2000; Knox et al. 2007; Sivasupramaniam et al. 2008) and environmental conditions (Dong and Li 2007; Martins et al. 2008). Seasonal variations, with concentrations in cotton leaves usually decreasing as the growing season progressed, were as large as 200 to 300% (He et al. 2006; Bird and Akhurst 2005; Kranthi et al. 2005; Olsen et al. 2005; Adamczyk et al. 2001; Adamczyk and Sumerford 2001). Consistent with these measurements, seasonal decreases in insect mortality on *Bt* plants have been observed in the laboratory (Bird and Akhurst 2005; Olsen et al. 2005) and in the field (He et al. 2006; Kranthi et al. 2005; Wu and Goa 2005). Conversely, Sayyed et al. (2003) reported >4-fold lower toxin levels in young *Bt* canola plants (4–5 week) compared to mature (7–8 week) plants, and Nguyen et al. (2007)

reported season-long increases in toxin concentrations in *Bt* corn. Genotype characteristics (*e.g.*, the site of insertion, gene construct, background genotype, epistasis, somoclonal mutation) have also been shown to influence toxin levels (Kranthi et al. 2005; Adamczyk et al. 2001; Adamczyk and Sumerford 2001; Sachs et al. 1998). And finally, different toxin levels have been measured in plants grown in different areas or under different environmental conditions (Dutton et al. 2004; Storer et al. 2001; Sachs et al. 1998; Martins et al. 2008).

Olsen et al. (2005) showed a decrease in transcript levels of the Cry1Ac gene as well as decreased protein levels in cotton. However, even late season plants retained high mortality for larval bioassays although toxin concentrations were reduced (control mortality using non-*Bt* leaves was high >95%) possibly due to low nutritional value of the late-season leaves or the presences of secondary metabolites. Similar results were shown by Gore et al. (2001) in a comparison of Bollgard, Bollgard II, and conventional cotton.

For pyramids, consider the situation in which one toxin is expressed a greater concentration than another in plant tissues. Kranthi et al. (2009) studied cotton containing Cry1Ac and Cry2Ab toxins. They found a ten-fold higher concentration of the Cry2Ab than the Cry1Ac in the plant tissues. This difference was greater late in the growing season when the Cry1Ac levels decreased. Consistent with these results, Mahon and Olsen (2009) fed *H. armigera*- resistant (RR, RS) and fully-susceptible to Cry2Ab on cotton containing Cry1Ac and Cry2Ab toxins and found that survival of all three genotypes was limited, but increased as the level of Cry1Ac decreased during the growing season. Approximately 8.5% of the *H. armigera* homozygous resistant to Cry2Ab completed pupation on Bollgard II cotton to adults. Survival of the homozygous resistant genotype is presumed to be because these insects have higher tolerance to Cry2Ab (Mahon and Olsen 2009).

11.9.3 Mode of Action

Understanding how *Bt* toxins work and how insects become resistant is the basis for developing strategies to delay the evolution of resistance. Individual *Bt* crystalline proteins (Cry) toxins are usually toxic to a limited number of species within an order and binding receptors on midgut epithelial cells have been shown to be critical determinants of their specificity. In general, the sequential steps in the Cry toxicity pathway are: (1) ingestion of the proteins by a susceptible insect larva, (2) solubilization of the protein in the insect midgut and protoxins are released, (3) cleavage of the protoxin by host proteases and release of the active toxin, (4) binding of the active toxin to specific receptors on the midgut epithelium, (5) oligomerization of toxin subunits to form pore structures that inject into the membrane, (6) passage of ions and water through the pores, resulting in swelling, cell rupture, and finally, insect death (Schnepf et al. 1998). In their review, Pigott and Ellar (2007) discuss the role of midgut binding receptors in Cry protein toxicity. The most characterized binding receptors have been for lepidopterans in which four classes of receptors have been identified: aminopeptidase N (APN) receptors, cadherin-like receptors,

alkaline phosphatases, and glycolipids (Pigott and Ellar 2007). Of the 38 different APNs described for 12 different lepidopterans, only 2 of 17 reported to bind Cry toxins have been shown to mediate toxicity. In contrast, cadherin-like receptors bind proteins and confer toxicity, which suggests that these proteins play a key role in toxicity, while APNs mediate pore formation, but do not have a direct role in toxicity (Pigott and Ellar 2007). There are three different mechanistic models to explain the Cry1A toxin mode of action: Bravo model, Zhang model, and the Jurat-Fuentes model (described in Pigott and Ellar 2007). The most widely accepted is the Bravo pore formation model, an updated version of the colloid-osmotic lysis model of Knowles and Ellar (1987), which proposed that Cry toxicity requires both binding to the cadherin receptor resulting in oligomerization of the toxin and binding to secondary receptors in the membrane such as APN receptors, which results in pore formation, lysis, and cell death (Bravo et al. 2004, 2007). The Zhang model proposes that receptor binding activates a signaling pathway involving stimulation of G protein, adenylyl cyclase, increased cyclic AMP levels, and activation of protein kinase A, leading to the formation of ion channels and subsequent cell death (Zhang et al. 2006). The Jurat-Fuentes model suggests that cytotoxicity is due to the combined effects of osmotic lysis (Bravo model) and cell signaling (Zhang model), in particular, activated Cry1Ac binds to a cadherin-like protein (HevCaLP), which results in the activation of an intracellular signaling pathway regulated by phosphatases (Jurat-Fuentes and Adang 2006). While binding receptors in lepidopterans have been the most widely studied, dipteran and coleopteran insects possess similar types of binding receptors, cadherins, APNs, and alkaline phosphatases, which suggests that these receptors are highly conserved across all three orders (Bravo et al. 2011).

11.9.3.1 Resistance Mechanism(s)

While there are other resistance mechanisms, only the binding receptor modification mechanism has a demonstrated causal link between the biochemical modification and Cry resistance (Ferré and Van Rie 2002; Ferré et al. 2007). Ferré and Van Rie (2002) indicate that in all cases of binding site modification, resistance is due to a recessive or partially recessive mutation in a major autosomal gene, and cross-resistance extends only to Cry proteins sharing binding sites. Cry proteins that do not share high levels of sequence similarity tend to have different binding sites and different modes of action. Resistance associated with modification of the binding site receptor on the midgut membrane has been described in a number of insect species (Ferré et al. 2008; Gahan et al. 2001; Heckel et al. 1997, 2007; Hernandez and Ferré 2005; Jurat-Fuentes and Adang 2006; Pigott and Ellar 2007). Cry1A resistance in TBW, PBW, DBM, and *H. armigera* has been linked to mutations in the cadherin genes, indicating a common genetic basis for resistance characterized by the properties of recessive inheritance, *i.e.*, >500-fold resistance to at least one Cry1A toxin, negligible cross-resistance to Cry1C, and reduced binding of membrane preparations to at least one Cry1A toxin (Heckel et al. 2007). For example, Gahan et al. (2001) showed that TBW Cry1Ac resistance was associated with disruption of a cadherin superfamily gene in a locus previously referred to as BtR-4.

Other *Bt* resistance mechanisms are not tied to mutations in the cadherin gene (reviewed in Tabashnik et al. 2011). These include alterations in proteases that cleave the protoxin, processing it into a smaller active toxin (Oppert et al. 1996; Candas et al. 2003), esterases that can bind and detoxify *Bt* toxins (Gunning et al. 2005), and loss or reduction of function of a putative 1,3-galactosyltransferase (Griffitts et al. 2001; Griffitts and Aroian 2005).

11.10 Assessing the Evolution of Resistance Through Simulation Modeling

Since the late 1990s, simulation models have helped guide EPA's regulatory decisions on the IRM requirements of both *Bt* cotton and *Bt* corn PIPs. Models used to simulate the potential for insect resistance development to *Bt* toxins were derived in large part from experience with resistance development to chemical pesticides (Gould 1998). These models have now been widely adapted to answer many questions surrounding the potential for resistance development to *Bt* PIPs, evaluate the relative success of potential resistance management strategies, and identify data gaps.

Simulation models have been developed to examine insect resistance evolution based on: (1) assumptions of the high-dose/refuge strategy, (2) estimations of the initial resistance allele frequency, (3) functional dose of the toxin, (4) understanding of the pest biology and ecology of the target pest, (5) population genetics, (6) population dynamics, and likely adoption of the *Bt* crop in the landscape, and (7) relative durability of IRM strategies (see for example Carrière et al. 2001a, 2004b; Glaser and Matten 2003; Vacher et al. 2003; Gould 1998). The high-dose/refuge strategy is expected to be the most effective if the dose of toxin received by the insect feeding on *Bt* plants is high enough to kill all or nearly all the heterozygotes. Under ideal circumstances, only rare, resistant individuals will survive a high dose produced by the *Bt* crop. Sensitivity analyses will reveal the most sensitive parameters to consider when evaluating resistance development and management strategies. Similarly, modeling will indicate data gaps, *e.g.*, larval movement, and lead to research in these areas in which there was sparse information.

The SAP has repeatedly stated that IRM models are important tools in evaluating the potential for insect resistance to *Bt* PIPs and in determining appropriate *Bt* crop IRM strategies (SAP 1998, 2001; also see Table 11.3). They agreed that models are the only scientifically rigorous way to integrate all of the available biological information, and that without these models, the EPA would have little scientific basis for choosing among alternative resistance management options.

While IRM models are very useful tools to study resistance evolution and resistance management, they represent an approximation of reality with uncertainties and limitations. Each model represents reality in a different fashion. Two important areas of uncertainty to consider are: (1) model structure, *e.g.*, depiction of time and space, representation of biological and ecological processes as explicit or implicit functions and (2) parameter uncertainty, *e.g.*, choice of parameters, input values, and parameter sensitivity. To use models in risk assessment, the results must be interpreted in light of model uncertainty (see Sect. 11.18).

Onstad (2008) describes the analysis of models as performing a scientific experiment. There are conditions that are held constant or conditions that are allowed to vary over time and space. Just as laboratory or field experiments need to be replicated, so do models to account for variation in the results. He describes six steps in the modeling process: (1) select the subject and purpose of the model, (2) review existing models and literature about experiments, (3) create mathematical functions from logic and data, (4) model verification, (5) model validation, and (6) analysis and experimentation.

For many simulations, the initial resistance allele frequency is chosen to reflect empirical findings or to facilitate model run time. The variables examined include those associated with insect biology and genetics, *e.g.,* toxin resistance mechanisms, resistance allele frequency, resistance allele expression, oviposition, dispersal, phenology, diapause, movement, population dynamics, population genetics, fitness costs, cross-resistance, and toxin sensitivity; those associated with the characteristics of the plants, *e.g.,* toxin levels, pyramiding, and plant defenses; and, finally, those associated with agricultural practices, *e.g.*, insecticide use, crop rotation and level of *Bt* crop adoption.

Empirical data is always the most desired option to parameterize a model. In the absence of data, modelers use scientifically-supported estimations to fully parameterize models. Scientists have examined many different biological, geographic, and spatial parameters that might reasonably be expected to have an influence on insect resistance development to *Bt* toxins expressed in transgenic crops. Many of these values were derived from published research of the biology and ecology of the target insects and from an understanding of *Bt* resistance from the study of laboratory colonies or insects that developed resistance to *Bt* microbial pesticides in the field. Past findings from studies concerning insect resistance to chemical pesticides, as contained in the 1986 NRC report (NRC 1986), have aided scientists in their understanding of resistance evolution to *Bt* plants. However, many parameter values have been estimations or based on assumptions, *e.g.*, genetics of resistance, functional dominance of the resistance allele, frequency of the resistance allele, fitness costs, cross-resistance potential, and mechanism of resistance. These were values for which empirical data did not exist or because they could not be measured in the absence of field resistance. At best, one could make assumptions about the inheritance of resistance or a likely mechanism of resistance. In other instances, the spatial and geographic scales needed for examining the population dynamics, for example, or for estimating the resistance allele frequency, were beyond those for which empirical approaches could be designed or they would be too costly or impractical.

11.11 Types of IRM Models

Mathematical models have proven to be very useful in evaluating how different biological and physical parameters might impact resistance development and how they might be utilized to slow or prevent such development. In the aftermath of the EPA reassessment of the risks and benefits of *Bt* PIPs in 2001, simulation modeling

has played a significant role in the development, evaluation, and requirement of insect-resistance-management strategies. IRM models have ranged from simple population genetic models that include only a few parameters (*e.g.*, Gould 1998) to much more complex models that may represent several interrelated complex process and incorporate potentially hundreds of parameters (*e.g.*, Storer et al. 2003a, b; Sisterson et al. 2004). Most IRM models begin with certain assumptions: resistance genes are autosomal (not on the sex chromosomes), single locus, and two alleles; resistance is recessive or functionally recessive (*i.e.*, moderate doses of the toxin are fatal to heterozygotes); and often mating between susceptible homozygotes and other genotypes is random. Models are used to examine evolution of resistance under different biological, genetic, and operational scenarios, *e.g.*, adult dispersal and oviposition, larval movement, with or without density-dependence, cross-resistance, different landscape patterns, resistance management options and the output may be expressed, for example, as changes to resistance allele frequency over a specific period of time, adaption rates over a specific period of time, and number of generations until a specific resistance allele frequency.

IRM models range from simple deterministic population genetic models that include only a few parameters to much more complex stochastic models that represent interrelated complex process and incorporate potentially hundreds of parameters (see reviews Caprio et al. 2008; Onstad 2008). As described by Peck (2000) and later echoed in Matten and Reynolds (2003), deterministic modeling, under discrete, non-random conditions, examines resistance evolution within a single field of *Bt* PIP crop to several thousand fields. On the other hand, stochastic, spatially-explicit, landscape modeling looks at the way random events may affect resistance evolution in multiple fields or with agricultural fields as patches in the landscape (*e.g.*, Storer et al. 2003a, b; Sisterson et al. 2004). Understanding the differences between deterministic and stochastic, spatially-explicit modeling is necessary to understand how the modeling results have been derived, what they mean, and how they may be used in evaluating a potential insect-resistance-management strategy. Whatever model is used, it is important to remember that each model has uncertainty associated with its predictions.

In addition to biological simulation modeling, several researchers have used economic simulation modeling to explore the implications of resistance mitigation strategies (Price et al. 2006; Goldberger et al. 2005), the potential impact of farmer adoption and willingness to accept mitigation strategies (Vacher et al. 2006; Linacre and Thompson 2003; Onstad et al. 2003; Hurley et al. 2001), and cost effectiveness of alternative resistance mitigation strategies (Crowder et al. 2005; Livingston et al. 2004). The results of economic modeling have been used by EPA in its evaluation of the likely implementation of resistance-management strategies by farmers based on acceptance of the technology, costs, and willingness to comply with refuge requirements (Berwald et al. 2006; Matten and Reynolds 2003; USEPA 2001). This chapter focuses on biological models, rather than economic models.

The following sections discuss the influence of biological, ecological, genetic, and operational factors on modeling of the evolution of resistance. The intent is to demonstrate their versatility as well as their limitations. For ease of reference, some significant findings from deterministic (Tables 11.5) and stochastic (Table 11.6) models are listed

Table 11.5 Use of deterministic insect resistance development models

Crop/Insect	Variables in simulations	Observations	References
Generic	Seed mixture, insect movement from plant to plant, selection factor, level of dominance	Under high levels of selection, low level of dominance, and intermediate to high levels of movement, seed mixtures may be worse than no resistance management strategy at all (Mallet and Porter); some mitigation with seed mixtures compared to "pure stands of toxic plants" (Tabashnik)	Tabashnik (1994b) and Mallet and Porter (1992)
Generic	Resistance allele frequency; heterozygote sensitivity to toxin; pyramid toxin with and without cross resistance allele	Resistance development is more susceptible to heterozygote mortality than initial resistance allele frequency; pyramiding has a distinct advantage over single toxin gene constructs in delaying resistance development; and smaller refuge size requirements are justified	Roush (1997, 1998)
Generic	Dominance and magnitude of resistance costs, refuge size, initial frequency of resistance alleles, and mode of resistance	Non-recessive costs of resistance, low initial resistance allele frequency, large refuges, incomplete resistance and density independent population growth in refuges favored prevention or reversal of resistance	Carrière and Tabashnik (2001)
Generic	Refuge structure, size and position in field, crop rotation (temporal refuge), insecticide use in refuges	Rate of resistance allele increase is lower with border refuges compared to infield random refuges; resistance allele frequency increased when refuges were sprayed with insecticides; higher rates of movement increased rate of resistance development; refuge positioning and size are important parameters for resistance management	Cerda and Wright (2004)
Generic	Egg mortality comparing density independent (DI), positively density dependent (PDD) or inversely density dependent (IDD)	The rate of resistance evolution is affected by egg mortality: High DI mortality delays resistance; comparatively evolution is fastest with PDD and slowest with IDD	Heimpel et al. (2005)
Generic	Level of toxicity in plants, initial resistance allele frequency, refuge size, and fitness cost associated with adaptation to toxins	Even small fitness costs associated with resistance alleles may have a large effect on the longevity of toxin-containing plants; If cultivars with one and two toxins are planted, resistance will develop	Gould et al. (2006)

Generic	Dominance of resistance alleles, impact of refuge when alternative insect hosts are in proximity	In the presence of an external supply of homozygous susceptible insects (*e.g.*, from unassociated alternative host plants), a refuge may increase resistance development	Mohammed-Awel et al. (2007)
Generic	Release of sensitive populations as pest control strategy in combination with resistance management	Insect release strategy seemed feasible over a wide range of environmental and biological conditions	Alphey et al. (2009)
Corn/ECB	Refuge structure, sequential planting, plant phenology, density independent overwinter mortality; density dependent larval mortality	Patchwork of transgenic and non-transgenic was preferable to uniform plantings for delaying resistance development; planting toxic variety first reduces insect density in nontoxic fields	Alstad and Andow (1995)
Corn/ECB	Seed mixture, refuge structure (block vs. row-strip), planting time, weather	Separate refuges delay resistance better than seed mixtures, small surviving fractions of heterozygous neonates reduces time to resistance 10–33 %, toxin titer decline may result in more rapid resistance development	Onstad and Gould (1998a)
Corn/ECB	Phenological relationships between the insect and host plants; modeled resistance development with progressive loss of toxin to zero at senescence	A decreasing toxin titer with the age of plants may reduce the time for resistance development	Onstad and Gould (1998b)
Corn/ECB	Dispersal, mixing, differences in male and female population sizes in transgenic and non-transgenic fields	Reducing dispersal between refuge and transgenic fields will decrease selection intensity for resistance alleles; assortative mating may reduce the mating success of males carrying resistance alleles; under conditions consistent with the high-dose/refuge strategy, spraying insecticides will have no effect on resistance evolution	Ives and Andow (2002)
Corn/ECB	Effectiveness of adaptive management strategies, monitoring strategies and cost analyses	Predicted resistance development could be prolonged by ten generations after detection of resistance alleles if refuge size is increased from 20 to 66 % or by decreasing survival and reproduction of ECB. Changing movement rates and attraction to Bt fields could prolong susceptibility for more than 20 generations	Andow and Ives (2002)

(continued)

Table 11.5 (continued)

Crop/Insect	Variables in simulations	Observations	References
Corn/ECB and SWCB	Refuge configuration, insect behavior	Insect behavior may be more important than refuge configuration or resistance allele expression; demonstrated behavioral differences between ECB and SWCB	Guse et al. (2002)
Corn/ECB, SWCB	Dispersal behavior of adults; refuge configuration	Insect behavior influenced resistance development more than refuge size; for ECB, block refuge or row-strip refuge resulted in similar resistance development predictions; for SWCB, block refuge was very effective, but row-strip not recommended	Onstad et al. (2002)
Corn/WCRW	Allele expression, toxin dose, crop rotation	Allele expression is most important factor; Medium toxin dose is most risky; higher doses keep resistance allele from increasing, lower doses ensure significant survival of susceptibles slows resistance development	Crowder and Onstad (2005)
Corn/NCR	Extended diapause; mating behavior; toxin dose	Product characteristics and farmer management practices (crop rotation, insecticide use) have a larger impact than population genetics parameters	Mitchell and Onstad (2005)
Corn/WCRW	Seed mixture, repellency, density dependence	Assuming a dominant R allele led to faster resistance development and assortative mating, seed mixtures had a positive effect (lower rate of evolution) on resistance development	Onstad (2006)
Cotton/CBW	Alternative (noncotton) plant hosts for *H. zea*; regional variation; fitness costs	Non-cotton hosts have played a role in suppression of *Bt* resistance development; the contribution of alternative hosts can be measured and included in determinations of “effective refuge” size; regional variation in “effective refuge” calculations should be considered in resistance monitoring strategies	Gustafson et al. (2006)
Cotton/CBW	Reproductive fitness on corn and cotton; dominance of resistant alleles, toxin levels in plants	Insecticides may be used to control *Bt* resistance evolution; moderate levels of toxin expression resulted in shortest resistance evolution time and high toxin levels resulted in the longest delay of resistance evolution	Ru et al. (2002)

Cotton/TBW	Refuge size and configuration, toxin dose, robustness of optimal strategies; take into account a fitness cost for resistance	In combination, the impact of different fitness cost parameters on model outputs are illustrated; increasing toxin dose results in concomitant decrease in refuge size as percentage of crop planted for sustainable control of resistance development	Vacher et al. (2003, 2004)
Cotton/PBW	Combinations of factors affecting resistance allele frequencies (refuge percentage, fitness cost, and incomplete resistance)	The direction of resistance allele frequency changes may vary over space and time relative to percent refuge, relative fitness costs, and mode of resistance	Tabashnik et al. (2005b)
Cotton/PBW	Diapause emergence rate, pesticide use, cultural control measures	Pesticide applications in refuges (non-Bt cotton) reduced the time to resistance establishment, cultural practices can affect resistance development (*e.g.*, time of planting vis à vis diapause emergence)	Carrière et al. (2001d)
Cotton/PBW	Contamination of refugia with Bt containing plants	Field measured levels of contamination had negligible effect on resistance development; simulations assuming high contamination (*e.g.*, 35%) resulted in accelerated resistance development predictions	Heuberger et al. (2008)
Cotton and Corn/ 6 insect pests[a]	Comparison of field monitoring data for resistance allele frequency and refuge area to model outputs; focus on relative dominance and fitness	The frequency of resistance alleles has increased in *H. zea,* but not in the other five major pests in Australia, China, Spain, and the United States; the dominance of the resistance allele is a major factor in the rate of resistance development; differences in the estimated "refuge abundance" for the six species is associated with a variety of biological parameters	Tabashnik et al. (2008)

CBW, TBW, ECB, PBW, *H. armigera, Seamia nonagrioides*.

Table 11.6 Use of stochastic insect resistance development models

Crop/insect	Variables in simulations	Observation	References
Corn/WCR	Refuge configuration, toxin dose	Infield block refuge is more likely to delay spread of resistance allele than separate refuge field at various locations; high dose will delay resistance.	Storer (2003)
Cotton and corn/CBW	A wide range of insect biological, genetic and plant variables were examined in sensitivity analyses	The relative dominance of resistance alleles, survival of susceptible insects on Bt crops, and the population dynamics influenced by developmental and environmental factors are the more important influences on resistance development; agricultural practices (*e.g.*, crop adoption rates, insecticide spraying) are also important considerations.	Storer et al. (2003b)
Cotton and corn/CBW	Spatial and temporal population dynamics, agronomic practices	Selection pressure is more intense in cotton with sprayed refuge than corn with un-sprayed refuge; initial gene frequency and functional dominance have a major impact on resistance evolution.	Storer et al. (2003a)
Cotton/CBW	Refuge size/configuration, age structure of adults and larvae, movement of larvae, migration among fields, plant type (including wild hosts)	Spatial and temporal patterns for refuges can have a strong effect on development of resistance; larval movement between Bt and non-Bt plants increased the rate of resistance development; if a resistance allele becomes established locally, it will spread rapidly throughout the planted region.	Peck et al. (1999)
Cotton/CBW	Larval survivorship, overwintering cost, dispersal, oviposition, refuge size	Intermediate levels of insect dispersal resulted in delayed resistance evolution relative to the ends of the range; similar results were found using a deterministic model although the latter predicted longer delays in resistance evolution; nonrandom mating increased resistance evolution, but coupled with nonrandom oviposition, resistance was delayed.	Caprio (2001)

Cotton/PBW	Cotton adoption vs. population densities of an ecological specialist over time	Models[a] predicted population decline as the proportion of Bt cotton planted in Arizona increased; combined with field data on PBW indicating such a decline in high use areas.	Carrière et al. (2003)
Cotton/PBW	Insect population size, carrying capacity	Time to resistance decreased as region size increased (increased probability of resistance establishing in a local area and spreading); carrying capacity can be an important determinant in resistance development.	Sisterson et al. (2004)
Cotton/PBW	Prolonged emergence from diapause; seasonal decline of Bt concentration in fruit; with and without spatial refuge	Prolonged diapause affected time to resistance, notes the potential for sub lethal effects of the toxin on insect development and fecundity may result a temporal refuge; relate slow adoption rate to lack of resistance development in Arizona but argue it is not sustainable even with refuge strategies	Gutierrez and Ponsard (2006)
Cotton/10 major pests	Sublethal effects, stenophagous highly susceptible pests vs. polyphagous highly tolerant pests; predator activity	Refuge strategy may be more appropriate for stenophagous than polyphagous insects; A refuge strategy designed for particular pest species may not be appropriate for other species; “insecticide use may differentially influence the development of resistance”	Gutierrez et al. (2006)

[a]Both a deterministic and stochastic model were used

and sorted by crop, *Bt* toxin, and pest. Many of the resistance development models are strategic models that are generally used to compare management designs. Some IRM models have been used to examine the evolution of resistance to *Bt* toxin sprays (*e.g.*, Caprio 1998a, b), but these have not been included in the tables.

11.12 Influence of Biological Factors on Resistance Evolution in Models

11.12.1 Impact of Delayed Development

Insect development is influenced by environmental factors including exposure to *Bt* toxins. The rate of larval development may be slowed when larvae consume only sub-lethal concentrations of toxin in plants (Gutierrez and Ponsard 2006; Liu et al. 1999, 2001a; Peck et al. 1999). Delayed larval development has implications regarding the probability of random mating. For example, assuming that some larvae are able to survive on *Bt* crops, but their development is slower than the larvae growing in refuge, then a temporal divide is created between when adults emerging from the *Bt* crop and the refuge population are ready to mate. This situation would likely lead to assortative mating because adult emergence is later on *Bt* plants than on non-*Bt* plants.

Delayed development of larvae could impact resistance development either positively or negatively. If delayed development led to reduced overwintering because the insects could not diapause or because sprayed defoliants removed their food source leading to loss of fitness then resistance development would be delayed. On the other hand, if defoliation comes late in the growing season, individuals experiencing delayed development may be more fit than those that develop normally. Additionally, the numbers of generations of insects during the growing season may impact the mating character of populations. In most models considering multivoltine populations, it is assumed that generation times do not overlap (Peck et al. 1999).

11.12.2 Population Dynamics

Area-wide suppression of ECB populations by *Bt* corn has been documented (Hutchinson et al. 2007, 2010). Similar results have been reported by Wu et al. (2008) for *H. armigeria*, and Carrière et al. (2003) for PBW. In the latter, the long-term suppression of PBW populations in Arizona was correlated to the adoption of *Bt* cotton. The authors used both a deterministic and a stochastic, spatially explicit model to simulate population effects and mapped annual PBW monitoring data collected both before and after *Bt* cotton adoption in 15 Arizona regions. The regional analyses demonstrated a suppression of the PBW population independent of weather and

geographic variation. Because a reduction in population size as demonstrated by Carrière et al. (2003) may be selective for heterozygotes, assuming they are less susceptible to *Bt* toxin than the homozygous susceptible insects, the resistance allele frequency may change. Sisterson et al. (2004) used a stochastic, spatially explicit model to explore the impact of pest population (PBW) size, carrying capacity, region size, and proportion of fields planted with *Bt* cotton on resistance development. Although these researchers found a high variance in the results, they concluded that the time to resistance (when the R allele frequency reached or exceeded 0.50) in their simulations was dependent upon a number of variables including population size, dispersal (the percentage of adults that leave their natal field) and the percentage of *Bt* PIP cotton fields. Increasing the percentage of *Bt* PIP cotton fields reduced the number of SS individuals available for mating, thus decreasing the time to resistance. Population size reduction reached a critical point when there were too few SS individuals available to prevent RR adults from mating with other RR or RS adults and thus R allele frequency dramatically increased (Sisterson et al. 2004).

Storer et al. (2003a) used a stochastic spatially-explicit model to examine the effect of spatial and temporal processes in the evolution of resistance in CBW in *Bt* PIP cotton and *Bt* PIP corn. The model suggests that selection for resistance is more intense in *Bt* PIP cotton fields than in *Bt* PIP corn fields and that local gene frequencies are highly dependent on local deployment levels of *Bt* PIP crops despite the high mobility of this pest.

11.12.3 Larval Movement

Larval exposure to *Bt* toxin is determined in part on how long the larvae feed on toxin-containing plants. For example, if heterozygote larvae are able to move from *Bt* plants to non-*Bt* plants and survive, there may be a preferential survival of less susceptible heterozygotes, and the frequency of resistance alleles will increase. Larger larvae are generally less susceptible to *Bt* toxins, heterozygote larvae that spend early life stages on non-*Bt* plants could move to and survive on *Bt* plants; thus, increasing heterozygosity. The SAP discussed four larval movement scenarios that could increase heterozygosity and thereby speed up the evolution of resistance (SAP 2011). These four scenarios are not mutually exclusive and could be easily incorporated into resistance evolution models.

1. The RS heterozygote larva hatches on a *Bt* plant, feeds a little bit, and then moves from a *Bt* plant to non-*Bt* plant where it completes development.
2. The RS heterozygote larva hatches on a non-*Bt* plant, and then late in life moves to a *Bt* plant, where it completes development.
3. The RS heterozygote larvae have a greater probability to move from a *Bt* plant to a non-*Bt* plant. Assume that here is no difference in individual survival probability between RS and SS larvae. Because more RS larvae move, then the RS survival rate is greater than the SS survival rate and heterozygosity will increase.

4. The late stage RS heterozygote larvae have a lower probability to move from a non-*Bt* plant to a *Bt* plant. Assume that here is no difference in individual survival probability between RS and SS larvae. In this case, more SS larvae will be exposed to *Bt* plants and die; therefore, there is a greater proportion of late instar RS heterozygote larvae that will survive and heterozygosity will increase.

Between-plant larval movement is of special concern in consideration of using seed mixtures as a resistance management strategy (Tabashnik 1994b; Mallet and Porter 1992; SAP 2011). Parker and Luttrell (1999) showed that TBW larvae moved between neighboring plants and that there was preferential movement from the *Bt* to non-*Bt* cotton. Additionally, Peck et al. (1999) concluded that larvae moving from *Bt* plants to non-*Bt* plants would result in higher levels of heterozygotes unless the cost of movement was high. Gore et al. (2002) reported significantly more movement on *Bt* cotton than non-*Bt* cotton. Tang et al. (2001) showed similar results for DBM on *Bt* and non-*Bt* broccoli in greenhouse tests. Hibbard et al. (2004) found significant movement of WCRW in corn fields: three plants down the row and across 0.76 m from one row to the next. Furthermore, neonate and later instar WCRW larvae preferred non-transgenic corn to Cry3Bb1 containing corn, a rationale why WCRW might move (Hibbard et al. 2005). Similar preferential movement away from *Bt* plants was shown for ECB by Davis and Onstad (2000).

11.12.4 Dispersal and Oviposition

The spatial location of a refuge is highly dependent on dispersal and oviposition of the target pest. Models of resistance evolution for high dose-refuge events generally find that the rate of evolution is slowest for intermediate levels of adult movement (Comins 1977; Caprio 2001; Ives and Andow 2002; Storer et al. 2003a, b; Sisterson et al. 2004; Ives et al. 2010). The evolution of resistance is fastest when adults do not move very much from their natal habitat (high degree of isolation affects mating and oviposition) or when they almost always move from their natal habitat (supply of purely susceptible insects will be reduced across the landscape), but it is slowest at intermediate rates of leaving (Ives et al. 2010). One explanation for this difference is based on the contrasting effect of male and female movement (Ives and Andow 2002; Hu and Andow 2011; Ives et al. 2010) and the implication of source-sink dynamics on the evolution of resistance.

For example, Caprio (2001) used both a stochastic, spatially explicit model and a deterministic model to look at the implications of source-sink dynamics and oviposition on the rate of resistance development. In this case, *sink* is defined as a habitat patch where the net reproductive rate of a population is less than replacement while a *source* is a patch with population having a net reproductive rate greater than 0. For *Bt* resistance development considerations, the source would be refuges and the sink would be the genetically modified crop. Using CBW and *Bt* cotton to parameterize

his models, Caprio suggested that non-random mating alone decreased the time to resistance, while non-random mating in combination with non-random oviposition delayed resistance development. In his simulations, Caprio included field-to-field dispersal rate. The higher the dispersal rate, the larger the ratio of the population size in refuges to the population size in the transgenic crop fields. Model simulations showed that intermediate dispersal rates of 1–10% per day resulted in a faster rate of resistance development compared to a higher (50%) or lower (0.1%) dispersal rate. Similar results were obtained by Ives and Andow (2002), Sisterson et al. (2004), and Ives et al. (2010). Predictions from the models simulating the possible effect of the degree of refuge isolation and oviposition were supported by empirical results on the behavior of CBW and TBW on cotton in the field (Caprio et al. 2004).

11.12.5 Insect Behavior

Differences in behavioral patterns between insects or within the same insect in different environments also influence resistance development. Guse et al. (2002) compared the simulated times for resistance development (number of years to reach 3% resistance allele frequency in the population) using standard assumptions of localized mating and oviposition for SWCB and random mating and uniform oviposition for ECB. Modeling indicated that the selective pressure for resistance development was much higher for the ECB than for SWCB and insect behavior had a greater impact on resistance development than did refuge size (Guse et al. 2002). The authors suggest that farming practices, *e.g.*, irrigation, refuge configuration, and placement could alter insect behavior patterns or influence them to improve resistance management efficacy (Guse et al. 2002). Crop management practices (*e.g.*, insecticide use and crop rotation) and *Bt* toxin dose had a greater predicted impact on resistance allele frequency than did biological or genetic factors for Northern corn rootworm (*Diabrotica barberi*, NCR) (Mitchell and Onstad 2005).

11.12.6 Impact of Alternative Hosts

Many insect pests utilize multiple non-*Bt* PIP hosts (crops and weeds), which do not express *Bt* toxins, as "natural refuge." China and India rely on alternative hosts to serve as natural refuge to delay the evolution of *H. armigera* and PBW resistance to *Bt* cotton. Prior to *Bt* cotton introduction in these countries, surveys were conducted to verify that there were sufficient alternative hosts available to serve as natural refuge where *Bt* cotton was grown (Wu et al. 2002; Ravi et al. 2005; Wu and Guo 2005). In the United States, alternative host evaluations were performed for CBW

(Stadelbacher 1979; Parker 2000; Kennedy and Storer 2000; Gould et al. 2002; Jackson et al. 2008; Head et al. 2010), TBW (Abney et al. 2004; Orth et al. 2007; Matten and Reynolds 2006; WCR (Oyediran et al. 2004; Wilson and Hibbard 2004; Clark and Hibbard 2004), and ECB (Tate et al. 2006).

Modeling played an important role in analyzing the contribution of CBW and TBW alternate hosts in delaying the evolution of resistance to *Bt* PIP toxin pyramids that are expressed in cotton. To date, these products include Bollgard II® cotton (Monsanto Company) which expresses both the Cry1Ac and Cry2Ab2 toxins, WideStrike® cotton (Dow AgroSciences, LLC) which expresses both the Cry1Ac and Cry1Fa toxins, and VipCot® cotton (Syngenta) which expresses both the VipAa19 and modified Cry1Ab toxins. Stable isotope analysis, ratio of C_{13} to C_{12}, was used to qualitatively infer host use based on whether adults come from larvae that had fed on C_3 plants (*e.g.*, cotton, soybean, peanuts, or tobacco) or C_4 plants (*e.g.*, corn, sorghum, or weedy, warm-season grasses). Gould et al. (2002) showed that in midsummer in Texas, less than 10% of CBW were developing on C_3 plants including cotton. Their results suggested that large portions (>50%) of late-season CBW fed on alternative C_4 hosts, primarily corn grown in the Corn Belt. The SAP (2004) recommended that more definitive data quantifying temporal and spatial production of susceptible CBW moths from each of the C_3 and C_4 hosts be collected. Subsequent to the 2004 SAP meeting, the USDA Agricultural Research Service and Monsanto conducted a thorough investigation of the utility of selected alternate hosts as refuges for the production of susceptible CBW, which included the following steps: (1) conduct field studies to evaluate CBW larval productivity on selected alternate hosts (*e.g.*, soybean, corn, peanut) (Jackson et al. 2005), (2) determine the availability of alternate hosts in the landscape, (3) perform a temporal analysis of CBW utilization of these hosts relative to cotton, (4) estimate effective refuge size, and (5) use IRM modeling to compare the relative durability of the 5% external structured non-*Bt* cotton refuge to the effective refuge of alternate hosts (Gustafson et al. 2006). The effective refuge size is the proportion of the overall insect population not exposed to the relevant *Bt* toxin(s) and was calculated using county-wide crop production data. Modeling results predicted that the relative contribution of alternate hosts to delay the evolution of resistance was comparable to the contribution of the 5% external, structured refuge. It is important to recognize that cropping patterns and available alternate crops are subject to change based on agricultural economic conditions; therefore, the durability of the alternative hosts as effective refuge may need to be reevaluated periodically.

In 2006, the question of whether alternative hosts crops (rather than use an external, 5% structured non-*Bt* cotton refuge) would be an effective refuge for TBW was discussed for *Bt* cotton pyramided with two *Bt* toxins. Rather than use stable isotope analysis to estimate the relative contribution of different hosts to local and regional hosts of TBW, host specific secondary metabolites were used (Orth et al. 2007). In particular, the cotton-specific secondary metabolite, gossypol, was used as well as the tobacco-specific secondary metabolite, cotinine. Gossypol levels were measured in TBW moths of various ages collected in 2004–2005

from alternate hosts in the vicinity of cotton fields in Arkansas, Georgia, Louisiana, Mississippi, North Carolina (also analyzed for cotinine levels), Tennessee, and eastern Texas (Jackson et al. 2008). The next steps in the investigation were identical to those performed for evaluating selected CBW alternate hosts as effective refuges, *i.e.*, spatial analysis of the availability of alternate hosts in the landscape, temporal analysis of TBW utilization of these hosts relative to cotton, calculation of effective refuge size, and modeling to compare the durability of alternate hosts as effective refuges with the durability of the 5% external, structured non-*Bt* cotton refuges to delay the evolution of resistance. The data and modeling were reviewed by EPA (Matten and Reynolds 2006) and presented to the SAP in June 2006. The SAP concluded that the provided data and modeling supported the conclusion that the proportion of TBW derived from non-cotton host plants was equal to or greater than that supplied by non-*Bt* cotton structured refuges to delay the evolution of resistance (SAP 2006).

The gossypol technique was used to determine whether alternative plant hosts would produce high numbers of CBW (Head et al. 2010). These analyses confirmed the results from the earlier studies performed using stable isotope analyses, but were more precise in quantifying the adults emerging from cotton versus other hosts.

11.13 Influence of Genetic Factors on Resistance Evolution in Models

11.13.1 Resistance Allele Frequency

The initial resistance allele frequency is a key parameter in predicting time to resistance development. The higher the frequency of the allele, the faster resistance is expected to occur (ILSI 1999; Andow and Hutchinson 1998; Gould and Tabashnik 1998). When doing sensitivity analyses on their resistance development model, Storer et al. (2003b) concluded that the initial resistance allele frequency is probably the most important biological parameter causing the greatest effect on the rate at which the population adapts to *Bt* PIPs. Crowder and Onstad (2005) came to a similar conclusion based on model runs for the WCR. Peck et al. (1999) simulated many different scenarios for resistance development focusing on TBW. Their results indicated that the higher the initial resistance allele frequency, the faster resistance developed. The greater the number of *Bt* fields, the greater the probability that resistant allele foci would develop and, once established, spread throughout a region. Alleles conferring resistance are typically rare, one in a thousand (Tabashnik 1994a; Gould et al. 1997; Burd et al. 2003; Tabashnik et al. 2008; Downes et al. 2009; Huang et al. 2009). A summary of estimated initial *Bt* resistance allele frequencies in pest populations unexposed to *Bt* toxins is shown in Table 11.7.

Table 11.7 Resistance allele frequencies in field

Pest (location)	*Bt* toxin	Assay method	Resistance allele frequency	Source
CBW (US)	Cry1Ac	Bioassay of isofemale lines (F_1) vs. Cry1Ac	0.00043	Burd et al. (2003)
	Cry2Aa	Bioassay of isofemale lines (F_1) vs. Cry2Ab	0.00039	
ECB (US)	Cry1Ab	F2 screen	0.0039	Andow et al. (2000)
ECB (France and US)	Cry1Ab	F2 screen	<0.00092 (France) <0.000423 (US)	Bourguet et al. (2003)
ECB (US)	Cry1Ab	F2 screen	<0.0044	Stodola et al. (2006)
PBW (US)	Cry1Ac	Bioassay of field-derived strains vs. Cry1Ac; field efficacy in Bt vs. non-Bt fields	0.004[a]	Tabashnik et al. (2000a, 2003b, 2005b)
PBW (US)	Cry1Ac	DNA-screening of field-collected individuals for cadherin mutations conferring resistance to Cry1Ac	Specific alleles not found using PCR	Tabashnik et al. (2006)
TBW (US)	Cry1Ac	Allelic recovery method	0.0015	Gould et al. (1997)
TBW (US)	Cry1Ac	DNA-screening of field-collected individuals for cadherin mutations conferring resistance to Cry1Ac	0.00007[b]	Gahan et al. (2007)
TBW (US)	Cry1Ac	F2 screen	0.0036–0.0263	Blanco et al. (2009)
OBW[c] (China)	Cry1Ac	Bioassay of field-derived strains vs. Cry1Ac	0 (Anci province) 0.00233 (Xiajian province)	Li et al. (2007)
OBW (Australia)	Cry1Ac	F2 screens (Mahon et al. 2007a, b) [Bioassay of field-derived strains vs. Cry1Ac (Downes et al. 2007)]	<0.0003 [0.0006]	Mahon et al. (2007b)
	Cry2Ab		0.0033 [0.0049]	Downes et al. (2007)
H. punctigera (Australia)	Cry1Ac	F2 screen	<0.0005	Mahon et al. (2009)
	Cry2Ab		0.0018	
Poplar leaf beetle (France)	Cry3Aa	Bioassay of field-derived strains vs. Cry3Aa	0.0049[d]	Wenes et al. (2006)
Diatraea saccharalis (US)	Cry1Ab	F2 screen	<0.0027[e] (major alleles) 0.0063 (minor alleles)	Huang et al. (2008)

[a]Mean of annual determinations from 1998 to 2004; in 1997 the frequency was reported as 0.16
[b]PCR analysis for a specific insertion sequence in >7000 field collected male TBW
[c]2002 to 2005 in two locations in China
[d]Pooled data from populations sampled from different regions in France at different times. *Bt* sprays had not been used in the areas that were sampled (*i.e.*, the populations had not been exposed to *Bt* toxin)
[e]With combined data from a previous study, the major resistance allele frequency was 0.001

11.13.2 Impact of Fitness Costs Associated with Resistance Alleles

Resistance adaptation may come at a cost to insects. A fitness cost occurs in the absence of the *Bt* toxin, *i.e.*, the fitness is lower for individuals with resistance alleles for *Bt* PIP resistance than for individuals without alleles for resistance. Fitness costs of *Bt* resistance take on many forms which effect life history traits such as egg viability (Groeters et al. 1994) and overwintering survival (Carrière et al. 2001b).

The consequences of including fitness costs in models of the evolution of resistance dramatically change the possible outcomes in four possible ways: (1) resistance could be delayed, (2) the population may become extinct, (3) the allele frequency could reach a stable equilibrium, (4) the population persists, but the allele is extirpated from the population (see discussion in SAP 2006). Several investigators have modeled the impact of *Bt* resistance on fitness (*e.g.*, Carrière and Tabashnik 2001; Gould et al. 2006; Tabashnik et al. 2005b; Zhao et al. 2005). For example, modelers demonstrated that resistance PBW had fitness costs that may extend the time to resistance (Carrière and Tabashnik 2001; Tabashnik et al. 2005b). Gould et al. (2006) completed simulations, which demonstrated that when toxin levels are high, even small fitness costs will prolong the time to resistance.

Recently, Gassmann et al. (2009) reviewed 70 studies of 14 species of moths and 3 species of beetles and found that fitness costs of toxin resistance were detected in 67% of the studies and in 34% of fitness component comparisons. Carrière et al. (2004a) determined that a plant's natural defenses, *e.g.*, gossypol production in cotton, increased the magnitude and dominance of some fitness costs. Experimental estimates of fitness costs could be incorporated in either deterministic or stochastic models to examine how such costs might influence resistance evolution. For example, Bourguet et al. (2000) used simulation modeling to examine the sensitivity of the evolution of resistance to the dominance of the resistance allele(s).

11.14 Influence of Operational Factors on Resistance Evolution Models

11.14.1 Impact of Toxin Susceptibility ("Dose")

Knowing the sensitivity of insects to the different *Bt* toxins is important for simulating realistic resistance development scenarios and establishing resistance management plans as well as for evaluating monitoring results. As mentioned above, the high-dose refuge strategy is predicated on having sufficiently high doses of toxin in plants such that heterozygotes (RS) are killed with high efficiency and the homozygous susceptible (SS) are killed completely. A high dose has been defined as 25 times the toxin concentration necessary to kill susceptible larvae (SAP 1998). This was considered to be a conservative definition derived from empirical toxicity

determinations and the assumption that resistance genes are functionally recessive (*i.e.*, SS and RS individuals are equally fit). But not all crop pests are highly susceptible to *Bt* toxins, and the doses in plants may be only moderately effective. In model simulations, insecticidal doses that allowed just 5–10% survival of susceptible homozygotes (SS) resulted in a high rate of resistance evolution (Onstad et al. 2001; Storer 2003; Crowder and Onstad 2005). The SAP indicated that survival of the heterozygote (RS) genotypes is the key driver for the evolution of resistance in models (SAP 2011). Often data are lacking and therefore it is not possible to use assumptions about SS survival (*i.e.,* dose determinations) to parameterize the model.

While TBW is quite susceptible to *Bt* toxins and consequently amenable to a high-dose/refuge strategy, CBW is only moderately susceptible to the Bt toxins used widely in corn and cotton (Storer et al. 2001). Building on the work of Peck et al. (1999), Storer et al. (2003a) did extensive modeling of CBW resistance development using parameter values specific to North Carolina. In their analyses, they concluded that selection is more intense in the more highly sprayed *Bt* cotton than in *Bt* corn, and increasing the percent adoption of Bt crops within a region led to rapid resistance development. Localized areas with high adoption rates and increased resistance allele frequencies would experience rapid spread of resistance through a region, which is consistent with the observations of Peck et al. (1999).

Tabashnik (1994a) summarized the variation in sensitivity to *Bt* toxin for 15 Lepidoptera and Coleoptera insect species. The results from between 2 and 13 populations of 14 different species and 49 populations of one species were reported. With the exception of DBM, the variation ratios (highest LC_{50} or LD_{50} for a given study divided by the lowest LC_{50} or LD_{50} from the same study, respectively) ranged between 1 and 42. DBM stood out with a variance ratio up to 700.

Annual *Bt* corn and *Bt* cotton resistance monitoring is a requirement of *Bt* PIP registrations. Characterizing the range of susceptibility of a pest to a *Bt* toxin is a prerequisite to initiating any resistance monitoring program. A baseline susceptibility range is the control for all future comparisons of susceptibility once an insect is exposed to the *Bt* toxins expressed in *Bt* crops. Significant changes in the susceptibility of an insect to a *Bt* toxin over time may be an indication of resistance. *Bt* toxin susceptibility studies have been published for CBW (Stone and Sims 1993; Ali et al. 2006; Jackson et al. 2006; Ali and Luttrell 2007), SWCB (Reed and Halliday 2001; Trisyono and Chippendale 2002), TBW (Stone and Sims 1993; Ali et al. 2006; Ali and Luttrell 2007); ECB (Maçon et al. 1999; Reed and Halliday 2001), WCR (Siegfried et al. 2005), CEW (Siegfried et al. 2000), PBW (Tabashnik et al. 2000b; Dennehy et al. 2004) and *H. armigera* (Li et al. 2007; Gujar et al. 2004, 2007; Chandrashekar et al. 2005). These studies have shown a range of sensitivities among the insect pests to the various Cry protein toxins (see Table 11.8). EPA has reviewed all of the Cry1Ab, Cry1F, Cry2Ab2, and Cry1A.105 resistance monitoring data for ECB, SWCB, CEW collected from 1996 to 2008 (USEPA 2010a, e). In addition, the Cry3Bb1, Cry34/35Ab1, and mCry3A resistance monitoring data for corn rootworm collected from the 2004 to 2008 growing seasons has been reviewed (USEPA 2010b, c, d). Similarly, EPA has reviewed the PBW, TBW, and CBW monitoring data for Cry2Ab, Cry1F, and Cry1Ac *Bt* cotton PIPs (public summaries in USEPA 2001; Matten 2006). Monitoring for PBW resistance to the Cry1Ac toxin and later

Table 11.8 Variable sensitivity to Bt toxins

Pest	Assay endpoint[a]	Toxin(s)	Variation ratio[b]	Reference
ECB	7 day, LC95	Cry1Ac	4	Marçon et al. (1999)
		Cry1Ab	6	
OBW[c]	96 h neonate LC50	Cry1Ac	>16	Gujar et al. (2007) and Chandrashekar et al. (2005)
		Cry1Ab	>12	
		Cry1Aa	>10	
ECB	LC90	Cry9C	<5	Reed and Halliday (2001)
WCR	4–7 day, LC50	Cry3Bb1	12 (field pop.)	Siegfried et al. (2005)
CEW	7 day, LC50 and LC90	Cry1Ab	<5	Siegfried et al. (2000)
CBW	7 day, LC50	Cry1Ab	130 (57 field pops.)	Ali et al. (2006)
TBW	7 day, LC50	Cry1Ab	12 (10 field pops.)	Ali et al. (2006)
CBW	7 day, LC50	Cry2Ab2	47	Ali and Luttrell (2007)
TBW	7 day, LC50	Cry2Ab2	17	Ali and Luttrell (2007)
SWCB	7–14 day LC50, LC95	Cry1Ab	38–46	Trisyono and Chippendale (2002)
CBW	7 day LC50	Cry1Ac	16 (15 strains)	Stone and Sims (1993)
TBW	7 day LC50	Cry1Ac	8 (12 strains)	Stone and Sims (1993)

[a]Similar results occur for development inhibition endpoints although the range may be smaller
[b]Highest reported LC50 or LD50 for a given study divided by the lowest LC50 or LD50 from the same study, respectively (Tabashnik 1994a)
[c]OBW = Old world budworm, *H. armigera*

Cry2Ab2 toxins has been conducted in Arizona since 1997 (summarized by Tabashnik et al. 2005a). Although some variation occurred from 1999 to 2003, the mean resistance allele frequency did not differ significantly between 1998 (0.007) and 2004 (0.004, 95% confidence limits 0–0.01) (Tabashnik et al. 2005a). DNA screening of 5 571 feral individuals collected from Arizona, California, and Texas during 2001–2005 detected none of the three cadherin alleles linked with resistance to Cry1Ac in several lab-selected strains of PBW (Tabashnik et al. 2006). The probability that the combined frequency of these three resistance alleles exceeded 0.0003 was less than 5%. Data from DNA screening, bioassays, and field efficacy tests have shown that PBW resistance to Cry1Ac remains low despite 10 years of commercial use of *Bt* cotton expressing the Cry1Ac toxin (Tabashnik et al. 2006).

11.14.2 Implications of Toxin Concentration Variability

Bt toxin concentrations within plants are known to vary substantially among the different plant tissues, in the growth stage of the specific tissues (*e.g.*, leaf), or within the entire plant (seasonal variation). Onstad and Gould (1998a, b) modeled the impact on resistance evolution in ECB of a gradual decline in toxin concentration as the plants aged, resulting in differential mortality patterns for homozygous susceptible insects and heterozygotes, which are less susceptible to the toxin. Given preferential survival of heterozygotes, the time for the resistance allele frequency to reach 3% in the population was shorter than under a scenario of constant toxin concentration. Gutierrez

et al. (2006) compared model results for stenophagous (restricted host range) insects and polyphagous insects and concluded that resistance is more likely to develop in the former than the latter. They suggested this could be due to the large effective refuge available to insects via alternative hosts or infield, temporal refuges as a result of increased survival of moderately *Bt*-susceptible larvae on plants coincident with seasonal reduction of the toxin concentrations in the plants.

11.14.3 Pesticide Use

Modeling the effect of insecticide applications in the refuge on the rate of evolution of resistance has been performed for a number of pests and crops. Ives and Andow (2002) predicted little or no impact of insecticide use on the evolution of resistance under high-dose conditions unless the susceptible ECB population was significantly reduced. On the other hand, when larval density dependence is taken into consideration, Heimpel et al. (2005) concluded that insecticide spraying in refuges would increase the rate of resistance evolution. Cerda and Wright (2004) concluded that non-*Bt* insecticide use resulting in 50–80% mortality in refuges would increase the rate of resistance evolution. Comparing ECB and SWCB, Onstad et al. (2002) suggested that when the resistance allele is recessive, spraying a refuge would not impact *Bt* resistance development in SWCB, but even one spray a year would increase the rate of resistance evolution in ECB. Mitchell and Onstad (2005) modeled resistance development in NCR. They predicted a ten-fold increase in resistance alleles if soil insecticides were applied to non-*Bt* corn refuges. Gutierrez et al. (2006) suggested insecticide use could differentially influence resistance development because of differences in insect biology, behavior, and sensitivity to toxin. Ru et al. (2002) predicted that the durability of *Bt* cotton could be doubled if 90% of *H. armigera* larvae on *Bt* cotton were killed with supplemental pesticides. Shelton et al. (2000) found in a greenhouse test, that spraying the refuge increased the rate of evolution of diamondback moth resistance. Gustafson et al. (2006) included pyrethroid treatments of *Bt* and non-*Bt* cotton for CBW control in their models. CBW larval mortality resulting from pyrethroid sprays on non-*Bt* and *Bt* cotton (Bollgard®), 64.6 and 62.8%, respectively (Greenplate 2004). Collectively, these studies indicate that insecticide use in the non-*Bt* crop refuge and *Bt* crop need to be considered as a factor in the evolution of resistance. The extent of the effect of insecticide use is dependent on insect biology and ecology, crop, and regimen of pesticide application.

11.14.4 Technology Adoption and Cost of Refuge

While this chapter focuses predominately on biological simulation models to predict the likelihood of resistance evolution, no IRM strategy can be successful without considering the economic implications of the strategy. Modeling efforts assume

that adoption of a given *Bt* PIP is 100%, a worse-case assumption. In reality, the actual adoption is likely to be significantly less than 100% and governed by multiple parameters, *e.g.*, availability of alternate controls, economic and market factors etc. Bioeconomic modeling has been used to explore the costs and benefits of resistance mitigation strategies (Price et al. 2006; Goldberger et al. 2005), the potential impact of farmer adoption and willingness to accept (comply with) mitigation strategies (Vacher et al. 2006; Linacre and Thompson 2003; Onstad et al. 2003; Hurley et al. 2001; Mitchell and Hurley 2006; Frisvold and Tronstad 2002), and cost effectiveness of alternative resistance mitigation strategies (Crowder et al. 2005; Livingston et al. 2004). Such modeling has aided EPA in its assessment of the benefits of *Bt* PIP technology, *e.g.*, environmental benefits, grower costs and willingness to comply with refuge requirements (Berwald et al. 2006; Matten and Reynolds 2003; USEPA 2001).

Hurley et al. (2001) found that high levels of *Bt* corn adoption reduces the evolution of resistance over a 15 year horizon and conditions of profit-maximization. In contrast, Frisvold (2006) developed a conceptual model to illustrate major issues in the choice of parameters, the tradeoff of the cost of actual (rather than optimal) refuge requirements versus resistance, *e.g.*, the short-run, annual costs of *Bt* cotton refuge requirements in the U.S., adoption behavior and refuge costs. Results indicate that the costs of refuges vary widely and single-year estimates are less reliable than longer-term averages. Langrock and Hurley (2006) used the contingent valuation method to characterize the sensitivity of farmer demand for corn rootworm *Bt* corn. While Secchi et al. (2006) modeled the use of a dynamic refuge to manage ECB resistance.

While biological models predict that increasing the refuge size would delay the evolution of resistance, larger refuges would increase the economic costs to the grower. Economic (including socio-economic) modeling suggests that there are real limits to the willingness of farmers to plant large refuges, especially when there are restrictions on using pesticides in these areas such that the crop yield is significantly reduced (Hurley et al. 2001, 2004; Andow and Ives 2002; Linacre and Thompson 2003; Onstad et al. 2003; Vacher et al. 2006). Pyramiding two toxins in crops is considered the approach most likely to delay development of insect resistance and encourage grower adoption because refuge size will be much lower.

Pyramiding multiple *Bt* genes in a crop plant is a strategy that will delay the evolution of *Bt* resistance and will reduce the cost of implementing a refuge because the size of the required refuge will much smaller than that for a single *Bt* gene product (Roush 1998; Gould 1998). The ideal situation is when all genes engineered into the plant are expressed at a high dose for all target pests and that each has an independent mode of action (low likelihood of cross-resistance). In effect, pyramiding multiple genes in the same plant will likely reduce the "push-pull" situation between mandating an IRM strategy and willingness to implement the IRM requirements.

As discussed earlier, design of a scientifically-based IRM strategy is based on the understanding of the biological, genetic, and operational factors that influence the evolution of resistance. IRM modeling assists in evaluating different IRM strategies in the context of the specific *Bt* PIP(s), crop, and primary insect pests. For example,

the IRM requirements for lepidopteran pests*, e.g.*, ECB, an above-ground foliar and stalk-boring corn pest, and for coleopteran pests, *e.g.*, CRW, a below-ground root-feeding corn pest are different based on evaluation of the risk factors. IRM requirements for lepidopteran pests of cotton, *e.g.*, TBW, CBW, and PBW, are different from those of corn and, in some cases, from each other.

The preferred resistance mitigation strategy in use over the past 15 years has been the high dose refuge strategy. Structured refuges provide a source of susceptible insects to mate with any rare, resistant individuals in the pest population to reduce the spread of resistance. The advent of two (or more) *Bt* gene expressed in the same crop plant to target the same insects, *i.e.*, pyramids, expanded the possible resistance management strategies for both *Bt* cotton PIPs and *Bt* corn PIPs. Beginning in 2007, EPA approved the use of natural refuges of alternative host plants to manage TBW and CBW resistance in two-toxin *Bt* cotton PIPs. In 2010, a seed mixture of 90% *Bt* corn seed and 10% non-*Bt* corn seed (often referred to as refuge-in-a-bag) was approved to manage CRW resistance (a separate 20% lepidopteran refuge was required). In 2011, a refuge-in-a-bag seed mixture of 95% *Bt* corn seed and 5% non-*Bt* corn seed was approved to manage both CRW and ECB (and other lepidopteran pests) in the same field. Given the growing number of *Bt* toxin combinations, the risk of resistance evolution will not be the same for all *Bt* PIP products and consequently, the IRM requirements will not be the same. EPA typically seeks the advice of the FIFRA SAP when there are major scientific issues concerning changes to IRM strategies (see SAP meetings listed in Table 11.3). The lesson is that IRM strategies (and requirements) need to be adaptive to the likelihood of resistance posed by specific *Bt* PIP products to the targeted pest complexes.

11.15 Refuge Size and Deployment

A structured refuge requirement includes the following: refuge size, refuge deployment (proximity of the refuge to the *Bt* PIP crop fields), and refuge management (acceptable chemical management of target pests in the transgenic fields and refuge, and agronomic management). With structured refuge, it is possible to specify and regulate the proximity of refuges to *Bt* crops.

Identifying the level of dose, as related to selection intensity, is crucial when determining the size and structure of a refuge needed to delay the evolution of resistance to a *Bt* PIP. If inheritance of resistance is recessive, the hybrid offspring produced by such matings will be killed by *Bt* crops, markedly slowing the evolution of resistance. Heterozygotes (those carrying one resistance allele) are functionally recessive. A high dose refuge strategy assumes that the *Bt* toxin will be expressed at high dose to cause >99.9% mortality of susceptible homozygotes and >95% of heterozygotes. If there is not a high dose, then there will be greater survival of resistant heterozygotes, in which case, the refuge size would need to increase to produce enough susceptible insects to dilute the percent resistance in the population (Roush

1997, 1998). The SAP has suggested that it is important to differentiate between high dose and non-high dose products when determining effective refuge size (*e.g.*, SAP 1998, 2001, 2002, 2009, 2011).

11.15.1 Pyramiding Toxins

A pyramiding strategy involves stacking two or more *Bt* genes together in a plant ("a pyramid") to express two or more *Bt* toxins with different modes of action to manage the evolution of resistance to the same insect spectrum. Such a tactic would result in "redundant killing" because each toxin would kill all insects susceptible to that toxin and kill all individuals that would be resistant to the companion toxin. Insects resistant to one of the toxins should remain susceptible to the second toxin. The probability of resistance to both toxins would be low because multiple genetic events would be needed to produce fully homozygous resistant individuals (four resistant alleles for two genes). In contrast to the high-dose refuge strategy used for single *Bt* toxin plants, the success of pyramided *Bt* toxin plants is dependent upon consistently high mortality of susceptible homozygotes by each toxin (Roush 1997, 1998). Several empirical studies have demonstrated the additive toxicity in cotton plants containing two *Bt* endotoxin genes (Greenplate et al. 2003; Jackson et al. 2004; Sachs et al. 1996).

Simulation models and greenhouse studies have shown that resistance evolves more slowly to two *Bt* toxins deployed as a pyramid than to either toxin independently (Mani 1985; Roush 1997, 1998; Caprio 1998a; Stewart et al. 2001; Tabashnik et al. 2002b; Zhao et al. 2003, 2005; Jackson et al. 2004; Bates et al. 2005; Gahan et al. 2005; Gould et al. 2006). Roush (1997, 1998) modeled the evolution of resistance to a single toxin expressed in a plant and to two toxins expressed in a pyramided *Bt* plant and showed an approximate ten-fold advantage to pyramiding in the absence of cross resistance. The same durability can be achieved using a smaller refuge in conjunction with a pyramid (Roush 1998). The advantages of a pyramid are contingent on several factors: high efficacy of each toxin against the target pests, independent segregation of resistance genes, and independent modes of toxicity against the target pests. In contrast to the high-dose refuge strategy needed for single-toxin plants, the success of two-toxin plants is dependent upon consistently high mortality of susceptible homozygotes by each toxin (Roush 1997, 1998).

These results are why the pyramiding of two *Bt* genes, for example, is so attractive as a resistance management tactic. This conclusion is also supported by other researchers who examined the economic benefits of managing resistance evolution to two toxins with dissimilar modes of action using a pyramided approach (Hurley 2000; Livingston et al. 2004).

When a pyramid is deployed makes a difference in its durability relative to a single toxin. In greenhouse experiments, concurrent use of transgenic broccoli expressing either Cry1C or Cry1Ac planted in close proximity to pyramided plants

expressing both Cry1Ac and Cry1C accelerated the evolution of DBM resistance; while, pyramided plants grown alone reduced the evolution of resistance compared to single toxin plants (Zhao et al. 2005). In addition, resistance will evolve first to the toxin that is being used singly. This same finding was reached by Gould et al. (2006) following their simulations of pyramided plants planted alone or along with plants having only one of the two toxins. Simulations showed that when the pyramided plants were grown alone, resistance alleles would reach a low frequency of equilibrium. However, when one and two toxin cultivars were grown as mixtures with a non-toxin cultivar, resistance to both toxins always evolved and fitness costs had little impact on the number of generations before a resistant population was attained. So, while cultivars with multiple toxins may delay resistance longer than cultivars with single toxins, that delay may not be significant if they are introduced in a mosaic with single toxin cultivars (Roush 1998; Hurley 2000; Zhao et al. 2005; Gould et al. 2006). This finding has important implications for the deployment of single *Bt* gene cultivars once pyramided *Bt* gene cultivars are commercialized. Because cross resistance will reduce any benefits gained from pyramiding, it is important to pay close attention to the choice of toxins.

Another consideration is the variation in toxin production throughout the growing season. For pyramids, consider the situation in which one toxin is expressed a greater concentration than another in plant tissues. Kranthi et al. (2009) studied cotton containing Cry1Ac and Cry2Ab toxins. They found a ten-fold higher concentration of the Cry2Ab than the Cry1Ac in the plant tissues. This difference was greater late in the growing season when the Cry1Ac levels decreased. Apparently consistent with these results, Mahon and Olsen (2009) fed *H. armigera*- resistant (RR, RS) and fully-susceptible to Cry2Ab on cotton containing Cry1Ac and Cry2Ab toxins and found that survival of all three genotypes was limited, but increased as the level of Cry1Ac decreased during the growing season. Approximately 8.5% of the *H. armigera* homozygous resistant to Cry2Ab completed pupation on Bollgard II cotton to adults. Survival of the homozygous resistant genotype is presumed to be because these insects have higher tolerance to Cry2Ab (Mahon and Olsen 2009).

11.15.2 Seed Mixtures

The proposal to use seed mixtures (*e.g., Bt* corn seed mixed with non-*Bt* seed) is attractive because it would ensure farmer compliance with the refuge. The refuge is "in the bag" so the grower does not have any indecision concerning whether a refuge should be planted or not. The science question is whether a seed mixture and separate refuges are comparable in delaying the evolution of resistance. Early modeling efforts indicated that seed mixtures delayed the evolution of resistance compared to separate stands, but this outcome was highly dependent on the degree of larval movement from plant to plant and the fitness cost of larval movement (Tabashnik 1994b; Mallet and Porter 1992). In 1998, EPA requested that the SAP provide advice on IRM strategies for *Bt* corn and *Bt* cotton (USEPA 1998). The SAP advised

EPA that seed mixtures were not a wise choice as an IRM strategy when there is larval movement between plants, *Bt* to non-*Bt* plants and vice-versa because RS heterozygotes will have a higher survival rate than when they are restricted to feeding only on *Bt* plants (SAP 1998). Ten years later, the SAP considered scientific issues associated with two specific *Bt* corn (with multiple PIPs) seed blend products (SAP 2009, 2011). At these meetings, the SAP considered many scientific questions including the ecological and evolution context of lepidopteran and CRW larval movement, adult dispersal and mating behavior, dose, and population dynamics (including density-dependent mortality). IRM considerations associated with a *Bt* PIP corn seed blend targeting corn rootworm, Optimum® AcreMax™1 Rootworm-Protected Corn (Pioneer Hi-Bred) were addressed in the February 2009 FIFRA SAP meeting. IRM modeling was developed to examine how seed mixtures affect the evolution of CRW resistance (Onstad 2006). In 2010, the SAP considered IRM questions with respect to SmartStax™ 1 Refuge-in-the-Bag (RIB) (a joint venture of Monsanto Company and Dow AgroSciences, LLC), a multi-trait PIP corn seed blend consisting of a mixture of 95% *Bt* corn seed and 5% refuge corn seed for IRM of above-ground lepidopteran targets, ECB and SWCB, and below-ground coleopteran targets, WCR, NCR, as the primary target pests. The SAP discussed four scenarios in which ECB larval movement could increase heterozygosity and thereby accelerate resistance evolution (SAP 2011). Pest-PIP-crop specific modeling was developed to study how ECB larval movement, for example, affected the durability of the *Bt* corn seed blends (Onstad and Gould 1998b; Davis and Onstad 2000). In *Bt* cotton, Agi et al. (2001) found that seed mixes were impractical because the plants sustained too much fruit damage and yield loss.

11.15.3 Alternate Hosts (Natural Refuge)

Beginning in 2004, IRM strategies for *Bt* PIP cotton began shifting from external, structured non-*Bt* cotton refuges to the use of alternate hosts as natural refuges to delay the evolution of TBW and CBW. IRM modeling was used to examine the relative contribution of CBW and TBW alternate hosts in delaying the evolution to *Bt* toxins expressed in Bollgard® (Cry1Ac) and Bollgard II® (Cry1Ac, Cry2Ab2) cotton, *Bt* PIP cotton products registered by Monsanto Company (Gustafson et al. 2006). Monsanto submitted data from multi-year field studies (ultimately published in Jackson et al. 2008), stable isotope analyses, host-specific metabolite analyses, spatial and temporal analyses of hosts, refuge calculations, and modeling to support the use of alternate hosts as natural refuge rather than a 5% structured non-*Bt* cotton refuge. The SAP reviewed EPA's analysis and agreed that alternative hosts were as durable as a 5% external structured non-*Bt* cotton refuge in delaying the evolution of TBW and CBW resistance to the Cry1Ac and Cry2Ab toxins expressed in Bollgard II® cotton, but were not as effective in delaying the resistance to the Cry1Ac toxin expressed in Bollgard® cotton (SAP 2004; 2006). Some cropping patterns were more effective in producing high numbers of susceptible insects on non-cotton hosts (*e.g.,* North Carolina) (SAP 2006). Based on the scientific

recommendations of the SAP, EPA removed the structured refuge requirements for TBW and CBW resistance management from the registration of Bollgard® II (later for Widestrike® cotton (Dow AgroSciences, LLC) and VipCot® cotton (Syngenta Crop Protection, LLC)) in the geographic areas bounded by eastern Texas to the Atlantic coast, but were kept in place for areas infested with PBW (USEPA 2011). In addition, all three structured refuge options remained in place for Bollgard® cotton due to the increased risk of the evolution of resistance in a natural refuge system (see discussion of modeling for single toxin and two toxin *Bt* cotton products in SAP 2006). When the natural refuge was approved as an amendment to the Bollgard® II cotton registration, the Bollgard® cotton registration was set to expire in late 2009. The use of alternative hosts rather than a structured refuge for *Bt* cotton resistance management marked a significant paradigm shift in the IRM requirements for *Bt* PIPs.

11.16 IRM Models Inform Monitoring Strategies

Monitoring to detect resistance alleles is a critical component in IRM plans for *Bt* PIPs. The goal of such programs is to have an early warning system to detect significant resistance allele shifts prior to resistance development in the field resulting in field failure. The success of resistance monitoring programs depends on having well-characterized baseline susceptibility data, rigorous sampling strategy, and sensitive detection methods. Resistance monitoring data are useful to look at susceptibility changes in populations collected at different locations. Monitoring results that show significant shifts in *Bt* PIP susceptibility could be an indication of the evolution of resistance in a population. Parameter values, such as resistance allele frequency for each target pest, susceptibility (indication of dose), selection intensity, and functional dominance could be changed based on monitoring results. Models would be rerun with these new parameter values and resistance management strategies could be modified prior to field-relevant resistance (field failure).

Using ECB as a model organism, Andow and Ives (2002) modeled how adaptation to significant shifts in susceptibility, an indication of resistant alleles in the sampled population, delays the evolution of resistance. Model results indicated that, at a minimum, resistance alleles should be detectable at frequencies of ≤1 in 5,000 to provide enough time (2 years) for implementation of an adapted IRM strategy. Andow and Ives (2002) provided three examples of how an existing IRM strategy could be adapted in light of significant susceptibility changes: (1) increasing the percentage of non-transgenic crop from 20 to 66%, (2) decreasing survival and reproduction of ECB from *Bt* corn fields by 90%, and (3) altering mating patterns via attraction of susceptible males into *Bt* corn fields. These authors concluded that early detection of resistance alleles combined with coordinated adaption of the resistance management strategies were predicted to increase the time to resistance by 10–20 generations.

Storer et al. (2003a) suggested using their model as a means for determining where to monitor for resistance development. Changing the level of adoption in the model will have an effect on the evolution of resistance. Sampling can be focused in geographic areas with high level of *Bt* PIP crop adoption where there is a higher probability of detecting resistance in field populations.

Tabashnik et al. (2008) analyzed more than 10 years of monitoring data from studies conducted in the United States, Australia, China, and Spain for CBW, *H. armigera,* TBW, ECB, PBW, and *Sesamia nonagrioides.* For each of the six pests, results of the monitoring studies were compared to results from computer modeling of resistance evolution. This analysis revealed that there was no substantial increase in resistance allele frequency for five of the six pests analyzed. Tabashnik et al. (2008) stated that the frequency of Cry1Ac resistance alleles had increased substantially in some field populations of CBW in the United States and this was evidence of resistance to the Cry1Ac toxin. The authors added that there was no evidence of field failure due other mitigating factors. Moar et al. (2008) wrote a rebuttal to dispute the analysis of the CBW monitoring data as evidence of resistance. At the heart of the discussion is the definition of resistance and relevance of laboratory analyses to field situations. Despite the disagreement in interpretation of the CBW data, there is agreement among scientists that planting refuges have helped to delay resistance (Huang et al. 2011).

11.17 Limitations of the Models

As noted previously, all models are approximations (simplifications) of reality and therefore, have uncertainties and limitations. For example, all models rely on certain assumptions (biological, genetic, spatial, and temporal). The power of the model and the degree of precision in the estimates they provide are linked to the uncertainty in the model structure (representation of the biological and ecological processes in time and space) and parameters (selection and values). A full understanding of modeling uncertainty is a reasonable prerequisite to using IRM models in risk assessment (see recent discussion in SAP 2011). EPA has published guidance on the development, evaluation, and application of models for environmental decision-making to increase transparency and improve the public's understanding of how science is used to make environmental decisions (USEPA 2009). This document is a useful reference for IRM model developers.

There are a variety of IRM models, which produce different results. Interpretation of the results depends on understanding the model structure and the underlying assumptions, both explicit and implicit, in the model. If more than one model is used in the risk assessment, then there are additional uncertainties to consider in making model comparisons. The EPA, Monsanto, and Dow AgroSciences built separate ECB and CRW IRM models to compare the durability of SmartStax™ Refuge-in-the-Bag (a mixture of 95% SmartStax™ corn seed and 5% non-*Bt* corn

seed) to a 5% structured refuge. The SAP discussed the model uncertainty of each IRM model used to predict the delay in the evolution of resistance and how uncertainty affects interpretation of the model output (USEPA 2011). One common source of uncertainty (decision model uncertainty) stems from considering only a limited range of models or comparisons among models, for example, a constraint on modeling efforts to compare a 5% seed mixture with a 5% structured refuge, but no other comparisons. There is also uncertainty about the structure of the models (model completeness). For example, models may leave out attributes that affect resistance evolution, such as density-dependent larval mortality. A further source of uncertainty that surrounds the predictions of the models is parameter uncertainty. A sensitivity analysis will highlight the parameters that most affect the outcome of a model. In the 2010 SAP meeting, the Panel emphasized that there was a high degree of uncertainty for pyramided *Bt* traits and seed mixtures, and recommended the use of sophisticated risk assessment techniques, such as scenario analysis and information gap analysis, to address these uncertainties (SAP 2011).

Modelers caution against making generalizations about models (Storer et al. 2003a, b; Caprio 2001). The number of different variables can be quite imposing and modelers should be aware of assumptions and default values used in simulations. The parameters selected may conform to a particular pest or pests, but not all pests. Model simulations crafted for one pest or crop will not necessarily be valid for other pests on the same crop or the same pest in other crops. Other factors that lead to uncertainty are time frame to delay resistance (*e.g.*, 10 years, 20 years, or indefinite), level of compliance, and level of adoption. Although the predictive capacity for models have limitations due to uncertainties, models are very useful for pointing the way to the most important parameters to consider when evaluating resistance development and management strategies. Models also give focus to empirical study intended to flesh out sparse datasets and to provide more robust input for future modeling efforts. In considering how model outputs (predictions) are used in decision making. Pielke (2003) offers six guidelines for consideration when using model outputs in decision making:

1. Predictions must be generated to meet the needs of the user.
2. Uncertainties must be clearly understood and articulated.
3. Those using model results should have experience with how models have been used and their success.
4. Alternative approaches should be fully considered.
5. Transparency (*e.g.*, providing opportunity for results to be questioned) should be ensured.
6. Reactions to the prediction process should be allowed for.

While limits on existing knowledge may constrain the certainty of modeling predictions, an increase in knowledge will not necessarily reduce uncertainty. Oreskes (2003) defines a relationship termed the complexity paradox: “The closer a model comes to capturing the full range of processes and parameters of the system being modeled; the more difficult it is to ascertain whether or not the model faithfully represents that system.” Gressel (2005) illustrates how differing assumptions may

result in large variation in model results paying particular attention, in the case of resistance development modeling, to the assumption of a single target for the toxin and that resistant individuals would behave in a manner that favors fitness characteristics.

11.18 IRM Program Requirements

Previous sections in this chapter provided a framework for assessing the likelihood of pest resistance evolving to *Bt* PIP crops and potential options to delay resistance. This section summarizes the IRM program requirements. The risk managers (in consultation with the risk assessors) make the regulatory decision on the specific IRM program requirements (see Fig. 11.2). Risk managers consider the scientific recommendations for managing the evolution of resistance with other factors such as grower compliance and environmental benefits (Andersen and Matten 2002). One of the difficulties in making a regulatory decision is that the perception of risk of the evolution of resistance differs among stakeholders. The challenge for a risk manager is how to balance the need to maintain the susceptibility of *Bt* through the institution of IRM requirements with the cost and willingness of the growers to comply with the IRM requirements. Ultimately, a successful IRM program will have to be based on the science, but needs to be cost-effective to be sustainable.

Since 1995, EPA has mandated IRM requirements intended to delay and mitigate the potential evolution of resistance. During this same period of time, registrants have developed various products using novel *Bt* toxins, and combinations of two or more *Bt* toxins expressed in the same plant to target the same set of insects (pyramided genes products) or different sets of insects (stacked gene products). There have been 40+ PIPs registered between 1995 and 2011 (Table 11.9). Of those, there are 20+ *Bt* corn PIPs and 3 *Bt* cotton PIPs currently registered for field use.

Bt PIPs are divided into five categories based on the number of toxins and pest targets, all five are applicable to currently registered *Bt* corn PIPs, but only one is applicable to currently registered *Bt* cotton PIP products (#3).

1. First generation PIP products which express a single *Bt* toxin.
2. Stacked *Bt* PIP corn products which express a single *Bt* toxin for control of lepidopteran pests of corn (*e.g.*, ECB), and also express a single *Bt t*oxin for control of coleopteran pests of corn (*e.g.*, WCR).
3. Pyramided *Bt* PIP products which express two or more *Bt* toxins with distinct modes of action for control of lepidopteran pests of corn or cotton.
4. Pyramided *Bt* PIP corn products which express two or more *Bt* toxins with distinct modes of action for control of lepidopteran pests of corn stacked with single PIP toxin for control of coleopteran insect pests.
5. Pyramided *Bt* corn PIP products which express two or more *Bt* toxins for control of lepidopteran corn pests stacked with two or more *Bt* toxins for control of coleopteran insect pests.

Table 11.9 List of plant-incorporated protectants registered from May 1995 to April 2011

Plant-incorporated protectant	Registrant	Date registered	Date expires
Bt potato Cry 3A	Monsanto 524-474	May, 1995	No expiration date
Bt corn Event 176 with Cry1Ab	Mycogen 68467-1	August, 1995	April 1, 2001
Bt corn Event 176 with Cry1Ab (2 products--field corn, popcorn)	Syngenta 66736-1	August, 1995; March, 1998	June 30, 2001
Bt cotton Cry 1Ac (Bollgard)	Monsanto 524-478	October, 1995	**Voluntarily cancelled** September 15, 2010
Bt corn Event MON 801 with Cry1Ab	Monsanto 524-492	May, 1996	**Voluntarily cancelled** May 8, 1998
Bt corn Event BT11 with Cry 1Ab (field corn – AgriSure CB and sweet corn - Attribute--no refugia for sweet corn)	Syngenta field corn 67979-1 sweet corn 65268-1	August, 1996; February, 1998	September 30, 2015
Bt corn Event MON 810 Cry1Ab (YieldGard)	Monsanto 524-489	December, 1996	September 30, 2015
Bt corn Cry 9C (domestic field corn for feed and non-food uses) (StarLink)	Aventis 264-669	May, 1998	**Voluntary cancellation** October, 2000
Replicase for potato leaf roll	Monsanto 524-498	November, 1998	No expiration date
Bt corn Event TC1507 with plant-optimized (PO) Cry1F (Herculex I)	Dow/Mycogen 68467-2	May 2001	September 30, 2015
Bt corn Event TC1507 with POCry1F (Herculex I)	Pioneer/Dupont 29964-3	May 2001	September 30, 2015
Bt cotton Cry2Ab2 in combination with Cry1Ac (Bollgard II)	Monsanto 524-522	December 2002	No expiration date
Bt corn Event MON863 with Cry3Bb1 (YieldGard RW)	Monsanto 524-528	February, 2003	September 30, 2010
Bt corn stack Events MON863+MON810 with Cry3Bb1+Cry1Ab (YieldGard Plus)	Monsanto 524-545	October 31, 2003	September 30, 2010
Bt cotton Cry1Ac+Cry1F (WideStrike)	Dow AgroSciences 68467-3	September 30, 2004	No expiration date
Bt corn Event DAS-06275-8 with MOCry1F (Mycogen Brand B.t. Cry1F Event DAS-06275-8 corn)	Dow AgroSciences 68467-4	May 27, 2005	September 30, 2015
Bt corn Event DAS-591227-7 with Cry34Ab1+Cry35Ab1 (Herculex RW)	Dow AgroSciences 68467-5	August 31, 2005	September 30, 2015
Bt corn Event DAS-59122-7 with Cry34Ab1+Cry35Ab1 (Herculex RW)	Pioneer/Dupont 29964-4	August 31, 2005	September 30, 2015

Bt corn Events DAS-59122-7 + TC1507 with Cry34Ab1 + Cry35Ab1 + PO Cry1F (Herculex Xtra)	Dow AgroSciences 68467-6	October 27, 2005	September 30, 2015
Bt corn Events DAS-59122-7 + TC1507 with Cry34Ab1 + Cry35Ab1 + PO Cry1F (Herculex Xtra)	Pioneer/Dupont 29964-5	October 27, 2005	September 30, 2015
Bt cotton Event MON531 with Cry1Ac (breeding nursery use only)	Monsanto 524-555	September 1, 2005	No expiration date
Bt cotton Event MON15947 with Cry2Ab2 (breeding nursery use only)	Monsanto 524-556	September 1, 2005	No expiration date
Bt corn Event MON88017 with Cry3Bb1 (YieldGard VT RW)	Monsanto 524-551	December 13, 2005	September 30, 2015
Bt corn Events MON88017 + MON 810 with Cry1AB + Cry3Bb (YieldGard VT Plus)	Monsanto 524-552	December 13, 2005	September 30, 2015
Bt Corn Event MIR 604 with modified Cry3A (Agrisure RW)	Syngenta 67979-5	October 3, 2006	September 30, 2015
Bt Corn Events MIR 604 + Bt11 with modified Cry3A + Cry1Ab (Agrisure CB/RW)	Syngenta 67979-8	January 24, 2007	September 30, 2015
Bt Corn Event Mon 89034 With Cry1A.105 + CryAb2	Monsanto 524-575	June 10, 2008	September 30, 2022
Bt Corn Events Mon 89034 + Mon 88017 With Cry1A.105 + Cry2Ab2 + Cry3Bb1	Monsanto 524-576	June 10, 2008	September 30, 2015
Bt Cotton Vip3Aa19 plus Modified Cry1Ab	Syngenta 67979-9	June 26, 2008	September 30, 2012
Vip3Aa20 (MIR162) in corn	Syngenta 67979-14	November 26, 2008	December 31, 2011
Bt Cry 1Ab (Bt11) + Vip 3Aa 20 (MIR 162) in corn	Syngenta 67979-12	Febuary 13, 2009	December 31, 2011
Bt Cry 1Ab (Bt11) + Vip 3Aa 20 (MIR 162) + modified Cry3A (MIR 604) in corn	Syngenta 67979-13	Febuary 13, 2009	December 31, 2011
(SmartStax) Bt Corn Events MON 89034 x TC1507 x MON 88017 x DAS-59122-7	Monsanto Company 524-581 Mycogen Seeds c/o Dow AgroSciences LLC 68467-7	July 20, 2009	November 30, 2011
Optimum AcreMax 1 (OAM 1) Seed Blend of Herculex Xtra + Herculex I	Pioneer/Dupont 29964-6	April 30, 2010	September 30, 2012
Optimum AcreMax RW (OAM RW) Seed Blend of Herculex RW + Non-Bt corn	Pioneer/Dupont 29964-6	April 30, 2010	September 30, 2012
C5 HoneySweet Plum (C5) Coat Protein Gene of Plum Pox Virus	USDA/ARS 11312-8	May 7, 2010	No expiration date

(continued)

Table 11.9 (continued)

Plant-incorporated protectant	Registrant	Date registered	Date expires
1507 (PO Cry1F) x MON 810 (PO Cry1Ab)	Pioneer/Dupont 29964-7	February 24, 2010	October 31, 2018
1507 (PO Cry1F) x 59122 (PO Cry34Ab1 + Cry35Ab1) x MON 810 (PO Cry1Ab)	Pioneer/Dupont 29964-8	February 24, 2010	October 31, 2015
59122 (PO Cry34Ab1 + Cry35Ab1) x MON 810 (PO Cry1Ab)	Pioneer/Dupont 29964-9	February 24, 2010	October 31, 2015
Bt Cry1Ac Soybean MON-877Ø1-2	Monsanto 524-594	September 9, 2010	September 30, 2012
(SmartStax) Bt Corn Events MON 89034 x TC1507 x MON 88017 x DAS-59122-7 – Seed Blend	Monsanto 524-595 Dow AgroSciences 68467-16	April 8, 2011	October 31, 2011

Each product category poses a different level of risk of evolution of resistance. Generally, a product with a single *Bt* gene will pose a greater risk for resistance than a product with two (or more) *Bt* genes as long as each toxin is produced at a high dose, each has a unique mode of action and there is a minimal likelihood of among the toxins. High dose products will pose less of a risk for the evolution of resistance than non-high dose products because of the low survival of resistant heterozygotes. In general, pyramided *Bt* PIPs will delay the evolution of resistance more than individual single *Bt* PIPs introduced sequentially or as a mosaic (Roush 1998; Zhao et al. 2003, 2005; Onstad and Meinke 2010). Refuge requirements for stacked *Bt* corn PIPs for corn borer and corn rootworm control add complexity to the design and deployment of the required refuge(s). Refuge strategies are determined by examining the different level of resistance risk between the two different sets of targets. Table 11.10 summarizes the refuge requirements for the different categories of *Bt* corn PIPs. Illustration of different deployment options for each type of refuge are found in the Seed Company Product Use Guides.

Over the past 15+ years, EPA has made changes to the IRM requirements of *Bt* PIP products to strengthen the refuge requirements, resistance monitoring, and compliance assurance programs, remain consistent with the state of the science of assessing the evolution of resistance, adapt to new *Bt* PIP technologies, and account for changes in regulatory policy. The basic IRM program elements for *Bt* PIP corn and *Bt* PIP cotton products were instituted in 2001 following the conclusion of a 2 year comprehensive *Bt* PIPs reassessment process (USEPA 2001).

1. Requirements relating to planting a refuge in conjunction with the planting of any acreage of a *Bt* PIP;
2. Requirements for the registrants to prepare and require users to sign “grower agreements” that impose binding contractual obligations on the grower to comply with the refuge requirements;
3. Requirements regarding programs to educate growers about IRM requirements;
4. Requirements regarding programs to evaluate and promote growers’ compliance with IRM;
5. Requirements regarding programs to evaluate whether there are statistically significant and biologically relevant changes in target insect susceptibility to the Cry protein in the target insects (includes annual reporting to the EPA);
6. Requirements regarding a “remedial action plan” that contains measures the registrants would take in the event that any field relevant insect resistance was detected as well as to report on activity under the plan to EPA;
7. Submit annual reports on units sold by state (units sold by county level will be made available to the EPA upon request), IRM grower agreements results, and the compliance assurance program including the education program.

The general language used to describe the requirements for each element is very similar across all *Bt* PIP products. This increases the uniformity and consistency in the IRM message received by the growers concerning the importance of implementing the IRM requirements and consequences should they be out of compliance. As

Table 11.10 Refuge requirements for *Bt* PIP Corn with corn borer and rootworm control traits

Bt corn PIP category and major pests	Refuge in corn belt	Refuge in cotton growing areas	In-field refuge deployment
Single *Bt* PIPs			
Corn borer	20 % refuge must be planted within ½ mile of the *Bt* corn borer protected hybrid. Refuge hybrids must not express a Bt protein active against corn borer	50 % refuge must be planted within ½ mile of the *Bt* corn borer protected hybrid. Refuge hybrids must not express a Bt protein active against corn borer	Refuge blocks or strips within a field must be ≥ 4 rows
Corn rootworm	20 % refuge must be planted in the same field or adjacent to the *Bt* corn rootworm protected corn hybrid. Can be separated by a ditch or a road, but not by another field. Hybrid must not express a Bt protein active against either corn rootworm	20 % refuge must be planted in the same field or adjacent to the *Bt* corn rootworm protected corn hybrid. Can be separated by a ditch or a road, but not by another field. Hybrid must not express a Bt protein active against either corn rootworm	Refuge blocks or strips within a field must be ≥ 4 rows
Stacked *Bt* PIPs – single *Bt* PIPs for corn borer and corn rootworm control	**Separate refuges are planted for corn borers and for corn rootworm:** **(a) Corn borer refuge:** 20 % refuge (50 % in corn/cotton growing areas) must be planted within ½ mile of the *Bt* corn borer protected hybrid. Refuge hybrids must not express a Bt protein active against corn borer **(b) Corn rootworm refuge:** 20 % refuge must be planted in the same field or adjacent to the *Bt* CRW-protected corn hybrid. Can be separated by a ditch or a road, but not by another field. Hybrid must not express a Bt protein active against corn rootworm **Common corn borer and corn rootworm refuge:** At least a 20 % (50 % in corn/cotton growing areas) refuge must be planted in-field or adjacent to the stacked trait field. Hybrid must not express a Bt protein active against either corn rootworm or corn borer		Refuge blocks or strips within a field must be ≥ 4 rows
Pyramided *Bt* PIPs with two or more *Bt* PIPs for corn borer	5 % refuge must be planted within ½ mile of the *Bt* corn borer protected hybrid. Refuge hybrids must not express a Bt protein active against corn borer	20 % refuge must be planted within ½ mile of the *Bt* corn borer protected hybrid. Refuge hybrids must not express a Bt protein active against corn borer	Refuge blocks or strips within a field must be ≥ 4 rows

Stacked and Pyramided multi-gene *Bt* PIPs for corn borer and corn rootworm	**Common corn borer and corn rootworm refuge:** Plant at least a 5 % (20 % in corn/cotton growing areas) refuge in-field or adjacent to the stacked trait field *Bt* corn borer protected hybrid. Hybrid must not express a Bt protein active against either corn rootworm or corn borer		Refuge blocks or strips within a field must be ≥ 4 rows
Seed Blends			
10 % Seed Blend – single toxin corn rootworm control only (no external corn rootworm refuge)	10 % hybrid that does not express a Bt protein active against corn rootworm in the bag + 90 % hybrid that does express a Bt protein against corn rootworm (all seeds in the blend express a single toxin for corn borer control – separate refuge needed for corn borers) **Plus separate corn borer refuge:** 20 % refuge (50 % in corn/cotton growing areas) must be planted within ½ mile of the *Bt* corn borer protected hybrid. Refuge hybrids must not express a Bt protein active against corn borer	Seed blend is not registered for use in corn/cotton growing areas	Seed blend refuge – no structured in-field corn borer refuge
5 % Seed Blend – pyramided multi-gene corn borer and corn rootworm control (No external structured refuges for corn borer or corn rootworm)	5 % hybrid that does not express a Bt protein active against corn borer or corn rootworm in the bag mixed with 95 % hybrid that does express multiple Bt proteins against corn borer and corn rootworm	Seed blend is not registered for use in corn/cotton growing areas	Seed blend refuge – no structured in-field refuge

there are many different *Bt* PIP products with different refuge requirements, it is important to have a clear set of IRM requirements. Annual resistance monitoring and compliance assurance reports provide EPA with information to assess the success of the IRM program.

11.19 Refuge Requirements

The size and structure of mandatory refuges has always been a hotly debated topic from the inception of *Bt* crop PIPs. Currently, there are three different types of required refuges for *Bt* PIPs expressed in corn and cotton.

1. Structured Refuge: A system of separate plantings of non-*Bt* plants used in conjunction with planting of *Bt* corn (or *Bt* cotton) fields to reduce the likelihood of insect resistance.
2. Natural Refuge: Alternative host plants may serve as a refuge in certain circumstances dependent on the pest, crop, and *Bt* trait. Currently, this option is only available for some two *Bt* gene cotton PIP products to manage TBW and CBW resistance.
3. Seed Mixture ("Refuge in a bag"): A mixture of a certain percentage of Bt PIP seed and non-Bt PIP seed. Currently, this option is only available for certain Bt corn PIP products.

11.19.1 Bt PIP Corn

IRM strategies for *Bt* corn PIPs have undergone multiple transformations since the first *Bt* corn PIP was registered for commercial use in 1995. At that time, there were no mandatory refuge requirements and no agreement on IRM strategies. The assumption was that adoption of this new technology would be slow in the first few years and selection for resistance would be low (see discussion in USEPA 2001; Andersen and Matten 2002). From 1995 to 1997, EPA granted time-limited, conditional *Bt* corn PIP registrations with voluntary refuge requirements of up to 20% in the Corn Belt. These registrations were set to expire on April 1, 2001. As part of the terms and conditions of these registrations, each registrant was required to submit a draft refuge strategy by August, 1998 and a final refuge strategy in January, 1999; this was extended to April, 1999. Each registrant was required to generate additional data to support a science-based IRM plan.

In 1998, EPA began to impose structured refuge requirements beginning with Cry9C field corn (Starlink® corn) making it the first registered *Bt* corn PIP with refuge requirements. These requirements were based on the EPA White Paper (USEPA 1998), SAP recommendations (SAP 1998), International Life Sciences International Report (ILSI 1999), The Union of Concerned Scientists report, *Now or Never* (Mellon and Rissler 1998), USDA, Cooperative State Research, Education,

and Extension Service and Agricultural Research Service NC-205 committee (NC-205 for short) published reports (Ostlie et al. 1997; NC-205 1998) and the published literature. Some other Cry1Ab field corn and popcorn products were registered by Novartis in 1998.

Beginning in 1996, the USDA NC-205 committee, whose function was to address research on the ecology and management of European corn borer and other stalk-boring Lepidoptera, began to discuss IRM strategies, in particular recommendations for the size and structure of the refuge for *Bt* corn PIPs (Ostlie et al. 1997; NC-205 1998). The NC-205 sponsored annual resistance management meetings, several symposia at annual Entomological Society of America meetings, and conferences to discuss IRM issues with scientists representing industry academia, USDA, and EPA as well as grower representatives from the NCGA and on occasion with members of public interest groups. These meetings provided opportunities for sharing information, establishing research priorities, and building trust among participants. The overall goal of the NC-205 efforts was to identify the best science-based and practical IRM strategies. It was the NC-205 efforts that led to a consensus approach to the structured refuge. At the same time the NC-205 was leading discussions on IRM strategies for *Bt* corn PIPs, the technology providers (registrants) formed their own group, Agricultural Biotechnology Stewardship Technical Committee (ABSTC). ABSTC members participated in all of the NC-205 meetings. In April 1999, ABSTC, in conjunction with the National Corn Growers Association, proposed uniform IRM requirements for *Bt* corn PIP registrations[3]. Cotton-growing regions represent a higher risk for resistance due to the potential double exposure of CEW to both *Bt* corn (Cry1Ab and Cry1F) and Bt cotton (Cry1Ac) during the same growing season. Modeling suggested that a sizable proportion of non-Bt corn (at least 50%) should be planted with *Bt* corn in *Bt* cotton growing regions to delay the evolution of resistance (Caprio 1998a).

Beginning with the 2000 growing season and later formalized in 2001 for all *Bt* corn PIP registrations, farmers were required to plant at least a 20% refuge that could be treated for insects, or a 50% treated refuge in cotton-growing areas[4] (EPA 2001).

[3] This plan represented a consensus effort between Novartis Seeds, Mycogen Seeds, Monsanto, Dekalb (now part of Monsanto), and the National Corn Growers Association (NCGA) for implementation in the 2000 growing season. The following Bt (Cry1A toxins) field corn registrations were covered under this plan: BT11 (EPA Reg. No. 67979–1), Event 176 (66736–1 and 68467–1), MON810 (524–489), and DBT418 (69575–1).

[4] Cotton-growing areas include the following states: Alabama, Arkansas, Georgia, Florida, Louisiana, North Carolina, Mississippi, South Carolina, Oklahoma (only the counties of Beckham, Caddo, Comanche, Custer, Greer, Harmon, Jackson, Kay, Kiowa, Tillman, Washita), Tennessee (only the counties of Carroll, Chester, Crockett, Dyer, Fayette, Franklin, Gibson, Hardeman, Hardin, Haywood, Lake, Lauderdale, Lincoln, Madison, Obion, Rutherford, Shelby, and Tipton), Texas (except the counties of Carson, Dallam, Hansford, Hartley, Hutchinson, Lipscomb, Moore, Ochiltree, Roberts, and Sherman),Virginia (only the counties of Dinwiddie, Franklin City, Greensville, Isle of Wight, Northampton, Southampton, Suffolk City, Surrey, Sussex) and Missouri (only the counties of Dunklin, New Madrid, Pemiscot, Scott, Stoddard) (USEPA 2001).

An external refuge must be deployed within 1/2 mile of a *Bt* corn field for lepidopteran pest control. In-field refuges had to be deployed as row strips at least four rows wide. The refuge could be treated with insecticides (but not *Bt* microbial sprays) based on economic thresholds. A discussion of all of the 2001 *Bt* PIP corn IRM requirements can be found in the *Bt* PIPs reassessment document (EPA 2001) with additional discussion in Glaser and Matten (2003) and Matten et al. (2004). These uniform requirements brought certainty and consistency to the market after the initial period where many *Bt* corn products had different refuge requirements.

Also in 2000, EPA decided to perform a comprehensive reassessment of the environmental risks and benefits of the registered *Bt* crop PIPs. EPA's draft reassessment was peer-reviewed by the SAP in October 2000 (SAP 2001). As a result of this reassessment, the IRM strategies for all *Bt* corn PIPs (only single trait PIPs were registered at that time) included a mandatory compliance assurance program, a more precise resistance monitoring program, and a few revisions to the structured refuge requirements.

The corn rootworm research community led by the USDA North Central Research Committee NCR-46, held similar discussions on IRM strategies for *Bt* corn PIPs for CRW control. The first *Bt* corn PIP for CRW control was registered in 2003 after considering the recommendations made by the SAP in August 2002 (SAP 2002). The CRW resistance management strategy included planting a 20% structured refuge adjacent to the *Bt* CRW-protected corn hybrids or as row strips at least 4 rows wide. Later in 2003, the first stacked lepidopteran and coleopteran *Bt* corn PIP was registered. The structured refuge requirements for *Bt* CRW-protected corn PIPs are different than for *Bt* lepidopteran-protected corn PIPs because of key differences in pest biology and ecology, dose (high dose versus non-high dose), population dynamics, and other biological, genetic, and operational factors discussed in this chapter.

In 2009 and 2010, the SAP reviewed the scientific issues associated with seed blend strategies (rather than separate structured refuges) consisting of non-*Bt* refuge seed mixed with *Bt* corn seed for control of CRW pests and lepidopteran pests (SAP 2009, 2011). Key factors in this discussion included biology and ecology of each pest, calculation of dose, role of density-dependent survival/mortality, consideration of high dose and non-high dose expression, larval and adult movement, mating behavior, and number of toxins (*e.g.*, pyramided or not). EPA registered the PIP products Optimum® AcreMax™1 (OAM1) B.t. Corn Seed Blend, a seed blend of 90% HERCULEX® XTRA (Cry1F, a lepidopteran-protective toxin and Cry 34/35Ab1, rootworm-protective toxins) and 10% HERCULEX® I (Cry1F), and OAM RW, a seed blend of 90% HERCULEX® RW and 10% Non-*B.t.* Corn in April, 2010 after consideration of the SAP's recommendations (SAP 2009). The first registered *Bt* corn PIP product to incorporate a "blended" refuge; *i.e.*, the OAM1 product requires a 10% refuge for corn rootworm, and the corn rootworm refuge seed is sold within the same seed bag as the *Bt* corn PIP rootworm product. Because all of the seed in the OAM1 blend is protected against lepidopteran damage because of the presence of Cry1F, OAM1 fields also require a separate 20% refuge for lepidopteran pests, and that refuge is in the form of a structured refuge that is sold

separately (see description of the IRM terms and conditions of registration, http://www.epa.gov/oppbppd1/biopesticides/pips/bt-seed-blends.pdf).

With respect to SmartStax® Refuge-in-the-bag (RIB) *Bt* corn,[5] a mixture of 5% non-*Bt* corn seed and 95% SmartStax® corn seed, the SAP expressed greater concern for the evolution of resistance by ECB and SWCB (both lepidopteran pests of corn) than CRW (coleopteran pests of corn) because of the difference in selection intensity to a high dose versus a low dose expression of *Bt* toxins (SAP 2011). This conclusion was the same as that reached by the 1998 SAP, for high dose cases when toxicity of the cultivar causes low survival of heterozygous pest individuals, seed mixtures will have lower durability than structured refuges with the same percentage of *Bt* plants (SAP 1998). The advice from the SAP was that resistance management for a pyramid should focus on the pest(s) with the greatest likelihood of resistance (SAP 2011). In this case, the SAP concluded that a *Bt* PIP seed and non-*Bt* PIP seed (refuge) is scientifically justified to manage the resistance of pests with limited larval mobility, *e.g.*, CRW, but is more debatable for ECB and SWCB because they have greater larval mobility and therefore, increased sublethal exposure moving from *Bt* to non-*Bt* plants in the same or adjacent rows. A general summary of refuge strategies required for *Bt* corn PIPs is shown in Table 11.10. A list of the registered *Bt* corn PIPs with their associated *Bt* toxins, target pests, and refuge requirements is shown in Table 11.11.

11.19.2 Bt PIP Cotton

The first *Bt* cotton PIP product Bollgard® cotton (Monsanto Company) was registered in 1995. Unlike the early *Bt* corn PIP products in which no structured refuge was required, EPA required a structured refuge be planted in close proximity to the Bollgard® cotton fields (a specific distance was not mandated) as a term of registration. A grower needed to plant either a 4% non-insecticide treated refuge or a 20% insecticide-treatable refuge or some combination on each farm. These requirements did not have specific deployment requirements. Early in the development of Bollgard® cotton, many organizations and scientists, *e.g.*, National Cotton Council, Arizona *Bt* Cotton Working Group, industry, EPA, and entomologists of the Cotton Insect Pest Management Forum recognized the significance of the risk of TBW, CBW, and PBW resistance to *Bt* cotton PIPs. In 2000, EPA decided to reassess the risks and benefits of *Bt* PIPs, which included Cry1Ac cotton (Bollgard® cotton). EPA used the advice of the SAP (SAP 2001), entomologists from USDA and academia (*e.g.*, see Hardee et al. 2001), growers, and other groups to evaluate different

[5] SmartStax® corn is a multi-toxin double pyramid in which there are three *Bt* toxins targeting lepidopteran stalk-boring (and ear feeding) pests (Cry1A.105, Cry2Ab2, and Cry1Fa) and two *Bt* toxins targeting corn rootworm (Cry34/35Ab1 and Cry3Bb1).

Table 11.11 *Bt* Corn trait groupings, *Bt* proteins, insects-.controlled or suppressed, and refuge requirements[a]

				Corn growing areas	Cotton growing areas
Trait group	Bt protein	Insects controlled[b] or *suppressed*	Herbicide tolerant[b,c]	Refuge % and location	Refuge % and location
Agrisure® [Syngenta; Syngenta + Dow AgroSciences]					
Agrisure CB/LL	Cry1Ab	**ECB** *CEW, FAW, SB*	LL	20 % –½ mile	50 % –½ mile
Agrisure GT/CB/LL	Cry1Ab	**ECB** *CEW, FAW, SB*	GT, LL	20 % –½ mile	50 % –½ mile
Agrisure RW	mCry3A	**CRW**	–	20 % –adjacent	20 % –adjacent
AgrisureGT/RW	mCry3A	**CRW**	GT	20 % –adjacent	20 % –adjacent
Agrisure CB/LL/RW	Cry1Ab mCry3A	**CRW, ECB** *CEW, FAW, SB*	LL	20 % –adjacent	50 % –adjacent
Agrisure 3000GT	Cry1Ab mCry3A	**CRW, ECB** *CEW, FAW, SB*	GT, LL	20 % –adjacent	50 % –½ mile
Agrisure Viptera 3110	Cry1Ab Vip3A	**BCW, CEW, ECB, FAW, WBC** *SB*	GT, LL	20 % –½ mile	50 % –½ mile
Agrisure Viptera 3111	Cry1Ab mCry3A Vip3A	**BCW, CEW, CRW, ECB, FAW, WBC** *SB*	GT, LL	20 % –adjacent	50 % –adjacent
Herculex® [Dow AgroSciences + DuPont /Pioneer]					
Herculex 1	Cry1F	**BCW, ECB, FAW, WBC**	LL, RR2 (some)	20 % –½ mile	50 % –½ mile
Herculex RW	Cry34/35Ab1	**CRW**	LL	20 % –adjacent	20 % –adjacent
Herculex XTRA	Cry1F Cry34/35Ab1	**BCW, CRW, ECB, FAW, WBC**	LL RR2 (some)	20 % –adjacent	50 % –adjacent
Optimum AcreMax® [DuPont/Pioneer]					
Optimum AcreMax1	Cry1F Cry34/35Ab1	**BCW, CRW, ECB, FAW, WBC**	LL, RR2	10 % in the bag (CRW) **plus** 20 % –½ mile (ECB)	50 % –adjacent[4]
Optimum AcreMax RW	Cry34/35Ab1	**CRW**	RR2	10 % in the bag	10 % in the bag

Yieldgard® [Monsanto]					
YieldGard CB (YGCB)	Cry1Ab	**ECB** *CEW, FAW, SB*	–	20 % –½ mile	50 % –½ mile
YieldGard VT	Cry3Bb1	**CRW**	RR2	20 % –adjacent	20 % –adjacent
YieldGard VT Triple (VT3)	Cry1Ab Cry3Bb1	**CRW, ECB***CEW, FAW, SB*	RR2	20 % –adjacent	50 % –adjacent
Genuity® [Monsanto]; SmartStax® [Monsanto and Dow AgroSciences]; SmartStax® with RIB Complete™ [Monsanto]; REFUGE ADVANCED™ powered by SmartStax®.[Dow AgroSciences]					
Genuity VT Double Pro (VT2P)	Cry1A.105 Cry2Ab2	**CEW, ECB, FAW**	RR2	5 % –½ mile	20 %–½ mile
Genuity VT Triple Pro (VT3P)	Cry1A.105 Cry2Ab2 Cry3Bb1	**CEW, CRW, ECB, FAW**	RR2	20 % –adjacent	20 % –adjacent
SmartStax or Genuity SmartStax (GENSS)	Cry1A.105 Cry2Ab2 Cry1F Cry34/35Ab1 Cry3Bb1	**BCW, CEW, CRW, ECB, FAW, WBC**	RR2, LL	5 % – adjacent (common refuge only, no separate corn borer and corn rootworm refuges)	20 % –adjacent
Genuity SmartStax with RIB Complete or REFUGE ADVANCED powered by SmartStax	Cry1A.105 Cry2Ab2 Cry1F Cry34/35Ab1 Cry3Bb1	**BCW, CEW, CRW, ECB, FAW, WBC**	RR2, LL	In the same bag: 5 % refuge corn seed mixed with 95 % SmartStax seed	Not used in cotton-growing areas

[a]Table adapted from Cullen and DiFonzo (2011). List of products is current as of April 30, 2011. Explanation of the refuge size and deployment are found in Table 11.9, Sect. 11.15

[b]Insects in **bold** are controlled. Insect in *italics* are suppressed. Insect abbreviations: *CW* black cutworm, *CEW* corn earworm, *CRW* corn rootworm, *ECB* European corn borer, *FAW* fall armyworm, *SB* stalk borer, *WBC* western bean cutworm

[c]**Herbicide traits listed in the table.** *GT* glyphosate tolerant, *LL* Liberty Link or glufosinate tolerant, *RR*2 Roundup Ready or glyphosate tolerant

refuge options described in the *Bt* PIPs Reassessment document (USEPA 2001). Following this reassessment, the following three refuge options were instituted:

1. *5% External, Unsprayed Refuge*. At least 5 acres of non-*Bt* cotton (refuge cotton) must be planted for every 95 acres of *Bt* PIP cotton. The size of the refuge must be at least 150 feet wide, but preferably 300 feet wide. This refuge may not be treated with sterile insects, pheromone, or any insecticide (except listed below) labeled for the control of tobacco budworm, cotton bollworm, or pink bollworm. At the pre-squaring cotton stage only, the refuge may be treated with any lepidopteran insecticide to control foliage feeding caterpillars. The refuge may be treated with acephate or methyl parathion at rates which will not control tobacco budworm or cotton bollworm (equal to or less than 0.5 lbs active ingredient per acre). The variety of cotton planted in the refuge must be comparable to Bt PIP cotton, especially in the maturity date, and the refuge must be managed (*e.g.*, planting time, use of fertilizer, weed control, irrigation, termination, and management of other pests) similarly to Bt PIP cotton. The non-*Bt* cotton refuge must be maintained within at least ½ linear mile (preferably adjacent to or within 1/4 mile or closer) from the Bt PIP cotton fields.
2. *20% External Sprayed Refuge.* At least 20 acres of non-*Bt* cotton must be planted as a refuge for every 80 acres of *Bt* PIP cotton (total of 100A). The variety of cotton planted in the refuge must be comparable to Bt PIP cotton, especially in the maturity date, and the refuge must be managed (*e.g.*, planting time, use of fertilizer, weed control, irrigation, termination, and management of other pests) similarly to *Bt* PIP cotton. The non-*Bt* cotton may be treated with sterile insects, insecticides (excluding foliar *Bacillus thuringiensis* subsp. *kurstaki, Btk,* products), or pheromones labeled for control of the tobacco budworm, cotton bollworm, or pink bollworm. The non-*Bt* cotton refuge must be maintained within at least 1 linear mile (preferably within ½ mile or closer) from the *Bt* PIP cotton fields.
3. *5% Embedded Refuge.* At least one single non-*Bt* cotton row must be planted for every six to ten rows of *Bt* PIP cotton. The refuge may be treated with sterile insects, any insecticide (excluding foliar *Btk* products), or pheromone labeled for the control of pink bollworm whenever the entire field is treated. The embedded refuge rows may not be treated independently of the surrounding *Bt* PIP cotton field in which it is embedded. The refuge must be managed (fertilizer, weed control, etc.) identically to the *Bt* PIP cotton.

There are three *Bt* PIP cotton products registered for commercial use: Genuity® Bollgard II® cotton (Monsanto Company), WideStrike® cotton (Dow AgroSciences, LLC), and VipCot® cotton (Syngenta Crop Protection LLC) (Table 11.12). Each product expresses a pyramid of two *Bt* toxins for control of TBW, CBW, PBW, and several other foliar-feeding lepidopteran pests. The registration of the single *Bt* toxin (Cry1Ac) Bollgard® cotton expired in 2010 (see Table 11.9). One of the key differences between the IRM requirements for *Bt* PIP cotton products and *Bt* PIP corn products is the use of natural refuge, alternate non-cotton host crops, to manage TBW and CBW resistance to two *Bt* gene, pyramided cotton products. A pyramided transgenic crop can offer advantages for IRM in that multiple toxins are expressed

Table 11.12 Summary of *Bt* PIP cotton products with refuge requirements

Bt Cotton Trade Name	*Bt* PIPs	Company	Structured refuge for pink bollworm control[a]	Natural refuge for tobacco budworm and cotton bollworm control[b]
Bollgard II®	Cry2Ab2+Cry1Ac	Monsanto Company	Yes	Yes
WideStrike®	Cry1Fa+Cry1Ac	Dow AgroSciences LLC	Yes	Yes
VipCot®	Vip3Aa19+Cry1Ab	Syngenta Crop Protection LLC	Yes	Yes

[a]**A structured refuge** is required in the states of Arizona, California, and New Mexico and in the following Texas counties: Brewster, Crane, Crockett, Culberson, El Paso, Hudspeth, Jeff Davis, Loving, Pecos, Presidio, Reeves, Terrell, Val Verde, Ward, and Winkler. No structured refuge requirements for PBW eradication programs. Check with local authorities regarding the exemption from refuge requirements.
[b]**A natural refuge** is permitted only in the following states: Alabama, Arkansas, Florida (except where restricted), Georgia, Kansas, Kentucky, Louisiana, Maryland, Mississippi, Missouri, North Carolina, Oklahoma, South Carolina, Tennessee, Texas (except Brewster, Crane, Crockett, Culberson, El Paso, Hudspeth, Jeff Davis, Loving, Pecos, Presidio, Reeves Terell, Val Verde, Ward, and Winkler), and Virginia.

simultaneously. This can decrease the likelihood of pest resistance development, since an insect resistant to one toxin will still be susceptible to the other provided that each toxin has a different mode of action and there is low cross resistance potential between the toxins. Monsanto provided data to support the production of susceptible CBW and TBW coming from alternate hosts as an effective natural refuge in comparison to a 5% structured non-*Bt* cotton refuge. The SAP reviewed EPA's analysis of the use of alternate hosts as a natural refuge at two SAP meetings, one focusing on the CBW (SAP 2004) and the other on TBW (SAP 2006). The SAP supported the use of alternate hosts as effective refuge for the production of susceptible TBW and CBW, but also recommended additional data be collected to confirm the availability and production of these insects throughout the cotton-growing regions primarily infested with TBW and CBW (not PBW). EPA reviewed the additional data submitted by Monsanto and natural refuge for resistance management of TBW and CBW to Bollgard® II cotton was approved in early 2007. This meant that no structured refuge was required to be planted in conjunction with Bollgard® II cotton products in cotton-growing areas predominantly infested with TBW or CBW (and some other lepidopteran pests), while a structured refuge was required in areas which are predominately infested with PBW (summarized in USEPA 2011). When a structured refuge is required, growers may choose from the three structured refuge options: 5% embedded, 5% external unsprayed or 20% external sprayed refuge. Later, two other two-toxin pyramided *Bt* cotton products, WideStrike® and VipCot®, were also approved for the use of natural refuge. The refuge requirements for currently registered *Bt* cotton PIP products are summarized in Table 11.12. Many counties

have exemptions from refuge requirements in accordance with ongoing PBW eradication programs in Arizona, New Mexico, California, and west Texas (Grefenstette et al. 2008). In 2006, the SAP provided EPA with the advice that the use of 100% Bollgard II® cotton as part of a PBW eradication program would not increase the risk of resistance (SAP 2007).

11.20 Monitoring Program

Monitoring to detect resistance is a critical component in an IRM program. Each *Bt* PIP registrant must have an annual resistance monitoring program and provide results to EPA annually. EPA requires that the *Bt* corn PIP registrants perform annual resistance monitoring for the lepidopteran corn pests: ECB, CEW, SWCB, and the coleopteran pests: CRW. The *Bt* cotton PIP registrants are required to have resistance monitoring programs for the following cotton insect pests: TBW, CBW, and PBW. Should field-relevant resistance be confirmed, an appropriate resistance management action plan will be implemented.

The resistance monitoring programs use two approaches: (1) focused population sampling and laboratory testing and (2) investigation of reports of less-than expected control of targeted insects. These collections and analyses of pest populations may reveal where resistance to a PIP is potentially developing and provide information on spatial locations of putative resistance "hot spots" in which further investigation is required. The success of a resistance monitoring program depends on having well-characterized baseline susceptibility data, sensitive detection methods, and an appropriate sampling strategy. These factors are weighed against the feasibility and cost of developing and implementing a resistance monitoring program (discussed in Andersen and Matten 2002). EPA requires that each resistance monitoring program include a detailed sampling plan and diagnostic bioassays for each of the targeted pests. Diagnostic or discriminating concentration assays were considered to be cost-effective detection methods for large-scale resistance monitoring efforts. Such assays are most effective when resistance is common or conferred by a dominant allele (resistance allele frequency >1%) (Andow and Alstad 1998). Resistance monitoring data are useful to verify parameter estimations in simulation models, such as initial resistance allele frequency for each target pest, susceptibility, selection intensity, and functional dominance or recessiveness of heterozygotes.

Several other resistance detection methods have been proposed over the years and each of these have strengths and weaknesses as discussed in Shelton and Zhao (2008) and Matten et al. (2004).These methods include: (1) systematic field surveys of *Bt* plants to monitor resistant phenotypes and gauge the geographic area where resistant populations exist (in-field detection), (2) the F_2 screen could be used to detect rare resistance alleles (Andow and Alstad 1998; Andow et al. 1998), although it needs further validation before it is widely accepted as a routine monitoring tool, (3) screening against resistant colonies (allelic recovery) could be used to identify recessive or incompletely dominant resistance alleles from field-collected males

(Gould et al. 1997) when there are previously isolated resistance alleles, (4) sentinel *Bt* crop field plots could be used to screen for resistant individuals (Venette et al. 2000); although, there is a concern with a high number of false positives; and (5) DNA-based screening used to detect resistant individuals with specific resistant alleles (Gahan et al. 2001; Tabashnik et al. 2006).

11.20.1 Resistance Monitoring for Lepidopteran Pests of Corn

The resistance monitoring program for lepidopteran-active *Bt* corn hybrids focuses on the following lepidopteran corn pests: ECB, *Diatraea grandiosella* (southwestern corn borer; SWCB), and *Helicoverpa zea* (corn earworm; CEW). The resistance monitoring program requirements for population sampling and investigations of unexpected damage were put in place as a result of the 2001 *Bt* PIPs reassessment (USEPA 2001). In 2010, a few modifications were made to this program (see "Terms and Conditions for Bt Corn Registrations, September 30, 2010," (USEPA, 2010f).

Population sampling for the target pests is focused in areas identified as those with the highest risk of resistance development (*i.e.*, where lepidopteran-active *Bt* hybrids are planted on a high proportion of the corn acres, and where the insect species are regarded as key pests of corn). Bioassay methods should be sensitive enough to detect field-relevant shifts in population response to the *Bt* toxin(s) expressed in the lepidopteran-active *Bt* hybrids and/or changes in resistance allele frequency in response to the use of the hybrids and, as far as possible, should be consistent across sampling years to enable comparisons with historical data. The number of populations to be collected shall reflect the regional importance of the insect species as a pest, and specific collection regions will be identified for each pest. For ECB, a minimum of twelve (12) populations across the sampling region will be targeted for collection at each annual sampling. For SWCB, the target will be a minimum of six (6) populations. For CEW, the target will be a minimum of ten (10) populations. Pest populations should be collected from multiple corn-growing states reflective of different geographies and agronomic conditions. To obtain sufficient sensitivity to detect resistance alleles before they become common enough to cause measurable field damage, each population collection shall attempt to target 400 insect genomes (egg masses, larvae, mated females, and/or mixed-sex adults), but a successful population collection will contain a minimum of 100 genomes. Often it is not possible to collect the target number of insect populations or genomes due to factors such as natural fluctuations in pest density, environmental conditions, and area-wide pest suppression. The sampling program and geographic range of collections may be modified as appropriate based on changes in pest importance and for the adoption levels of the lepidopteran-active *Bt* corn hybrid. The registrant must consult with EPA before implementation of such modifications. The results of the population sampling and bioassay monitoring program are reported annually to EPA. Any incidence of unusually low sensitivity to the *Bt* protein used in bioassays, *e.g.*, Cry1Ab, Cry1Fa, is investigated as soon as possible to understand whether there is resistance

is relevant to field performance. Such investigations should proceed in a stepwise manner until the relevance of the resistance to field performance can be either confirmed or refuted, and results of these shall be reported to EPA annually.

The investigative steps include the following:

1. Re-test progeny of the collected population to determine whether the unusual bioassay response is reproducible and heritable. If it is not reproducible and heritable, no further action is required.
2. If the unusual response is reproducible and heritable, progeny of insects that survive the diagnostic concentration will be tested using methods that are representative of exposure to the lepidopteran-active *Bt* corn hybrid under field conditions. If progeny do not survive to adulthood, any suspected resistance is not field-relevant and no further action is required.
3. If insects survive steps 1 and 2, resistance is confirmed, and further steps will be taken to evaluate the resistance. These steps may include the following: determining the nature of the resistance (*i.e.*, recessive or dominant, and the level of functional dominance); estimating the resistance allele frequency in the original population; determining whether the resistance allele frequency is increasing by analyzing field collections in subsequent years sampled from the same site where the resistance allele(s) was originally collected; determining the geographic distribution of the resistance allele by analyzing field collections in subsequent years from sites surrounding the site where the resistance allele(s) was originally collected. Should field-relevant resistance be confirmed, and the resistance appears to be increasing or spreading, the registrant will consult with EPA to develop and implement a case-specific resistance management action plan.

A second portion of the resistance monitoring program is investigating all legitimate reports of unexpected levels of damage by target pests reported by growers, extension specialists, or consultants. If reports of unexpected levels of damage to corn lead to the suspicion of resistance in any of the key target pests (ECB, SWCB, and CEW), then a series of steps are taken to investigate whether or not there is resistance. Suspected resistance is defined by unexpected levels of insect-feeding damage for which the corn in question has been confirmed to be lepidopteran-active *Bt* corn; the seed used had the proper percentage of corn expressing the *Bt* protein; the relevant plant tissues are expressing the expected level of the *Bt* protein; and it has been ruled out that species not susceptible to the protein could be responsible for the damage, that no climatic or cultural reasons could be responsible for the damage, and that there could be no other reasonable causes for the damage. If resistance is suspected, the registrant (seed company representatives) instructs the growers to do the following: Use alternative control measures in their *Bt* fields in the affected region to control the target pest during the immediate growing season. Destroy *Bt* crop residues in the affected region within 1 month after harvest with a technique appropriate for local production practices to minimize the possibility of resistant insects over-wintering and contributing to the next season's target pest population. Additionally, if possible, and prior to the application of alternative control measures or destruction of crop residues, the registrant will collect samples of the insect

population in the affected fields for laboratory rearing and testing. Such rearing and testing shall be conducted as expeditiously as practical.

EPA defines confirmed resistance to mean, in the case of field reports of unexpected levels of damage from the key target pests, that all the following criteria are met: There is >30% insect survival and commensurate insect feeding in a bioassay, initiated with neonate larvae, that uses methods that are representative of exposure to Bt corn hybrids under field conditions (ECB and SWCB only). In standardized laboratory bioassays using diagnostic concentrations of the *Bt* protein suited to the target pest in question, the pest exhibits resistance that has a genetic basis and the level of survivorship indicates that there may be a resistance allele frequency of ≥ 0.1 in the sampled population. In standardized laboratory bioassays, the LC50 exceeds the upper limit of the 95% confidence interval of the LC50 for susceptible populations surveyed both in the original baselines developed for this pest species and in previous years of field monitoring.

11.20.2 Resistance Monitoring for Coleopteran Pests of Corn

Registrants must monitor for CRW (western CRW and northern corn rootworm, *Diabrotica barberi*) resistance and/or trends in increased tolerance to the *Bt* toxin. The resistance monitoring program for CRW-active *Bt* corn hybrids is structured similarly to that for lepidopteran-active *Bt* corn hybrids. Sampling is focused in those areas in which there is the highest risk of resistance development, typically in high adoption areas. The resistance monitoring plan includes the following: baseline sensitivity data, sampling (number of locations, samples per locations), sampling methodology and life stage sampled, bioassay methodology and sensitivity, standardization procedures (including quality assurance/quality control provisions), statistical analysis of the probability of detecting resistance, and a description of rootworm damage guidelines. A diagnostic assay is used to detect potentially resistant CRW, *e.g.*, the Sublethal Seedling Assay (Nowatzki et al. 2008). The registrant must follow-up on grower, extension specialist, or consultant reports of unexpected damage or control failures for corn rootworm. Any suspected resistance must be investigated. If corn rootworm resistance is confirmed, all acres of the CRW-active *Bt* corn hybrid and refuges must be treated with insecticides targeted at CRW adults and larvae. A remedial action plan must be implemented in the affected area following EPA approval.

11.20.3 Resistance Monitoring for Lepidopteran Pests of Cotton

Each *Bt* cotton PIP registrant is required to have a resistance monitoring program to detect insect resistance as early as possible. The resistance monitoring program for *Bt* cotton PIPs is structured similarly to that for lepidopteran-active *Bt* corn hybrids.

Like *Bt* corn PIPs, the resistance monitoring program for *Bt* cotton PIPs has two parts: population sampling and investigations of unexpected damage (see 2001 *Bt* PIPs reassessment, USEPA 2001). The resistance monitoring plans focus on TBW, CBW, and PBW. The monitoring program description includes: baseline sensitivity data, sampling (number of locations, samples per locations), sampling methodology and life stage sampled, bioassay methodology and sensitivity, standardization procedures (quality assurance/quality control provisions), and statistical analysis of the probability of detecting resistance. Collection sites should be focused in areas of high adoption of the *Bt* cotton PIP to cover the range of TBW, CBW, and PBW. Baseline susceptibility to the *Bt* protein(s) are need for TBW, CBW, and PBW. The registrant must follow-up on grower, extension specialist, or consultant reports of unexpected damage or control failures, *e.g.*, such as increases in damaged squares or bolls. Any incidence of unusually low sensitivity to the *Bt* protein used in bioassays, *e.g.*, Cry1Ac, Cry2Ab2, Cry1Fa, is investigated as soon as possible to understand any field relevance of such a finding. The following testing scheme is used to confirm or refute the field relevance:

1. Determine if the observed effect is heritable;
2. Determine if the increased tolerance can be observed in the field (*i.e.*, survive on *Bt* cotton plants);
3. Determine if the effect is due to resistance and if resistance is confirmed,
4. Determine the nature of resistance (dominant, recessive),
5. Determine the resistance allele frequency,
6. Determine, in subsequent years, whether the resistance allele frequency is increasing, and
7. Determine the geographic extent of the resistance allele (or alleles) distribution.
8. Should field-relevant resistance be confirmed, and the resistance appears to be increasing or spreading, the registrant will consult with EPA to develop and implement a case-specific remedial action plan.

11.21 Remedial Action Program

A remedial action plan is required for suspected and confirmed resistance. The remedial action plan defines the steps needed to investigate suspected resistance when there is any incidence of unusually low sensitivity to the *Bt* protein(s) in bioassays or if reports of unexpected levels of damage lead to the suspicion of resistance in any of the key target pests. Should resistance be confirmed and considered to be field-relevant (field failure) then additional actions are required. The first step once resistance has been confirmed is to notify EPA, farmers involved to treat their *Bt* crop with alternative pest control measures. This might be a chemical pesticide known to be highly effective against the insect or it might mean measures such as crop destruction. In addition, the sale and distribution of the *Bt* PIP crop would be suspended in that area and the surrounding area until an effective local mitigation plan, approved by EPA, has been implemented. The registrant and other stakeholders

develop the case-specific resistance management action plan. For example, if resistance is "suspected" for PBW then the registrant implements the Arizona Bt Cotton Working Group's Remedial Action Plan to address that situation. Other registrants with the same (or similar) *Bt* PIPs would be notified. There would also need to be increased monitoring to define the remedial action area(s). Geospatial survey would help define the scale of remedial action and where to intensify monitoring. Possible remediation tactics include: increasing refuge size, changing dispersal properties, use of sterile insects, or use of other modes of actions. These tactics, for the most part, are untested. The ideal situation would be that resistance is reversible and insects in the affected area would "regain" their susceptibility to the *Bt* proteins. Remediation efforts would be reported to EPA as part of the annual resistance monitoring program report. The greatest concern with remedial action plans is that they will not work either to eradicate resistance or mitigate it. This concern was noted by the 2000 SAP Subpanel (SAP 2001).

11.22 Grower Education and Compliance Assurance Program

Grower compliance with IRM requirements is essential to the success of any resistance management program. Non-compliance with these requirements will likely increase the evolution of insect resistance to *Bt* PIPs crops and potentially compromise the environmental and grower benefits of this technology. Following the *Bt* PIPs reassessment in 2001, each registrant was required to institute a comprehensive grower education program stressing the importance of the IRM program and a compliance assurance program to evaluate the extent to which growers are complying with the IRM program and actions to address non-compliance (USEPA 2001). The compliance assurance program is designed to promote grower compliance with refuge requirements, irrespective of farm size. The program is composed of several parts: grower agreements that legally bind growers to follow the IRM requirements, annual grower affirmation of IRM requirements, comprehensive grower education program, third-party telephone survey of growers to look broadly at compliance on a regional levels, on-farm assessments, follow-up actions using the phased compliance approach should non-compliant growers be identified through the on-farm assessment program, and a tips and complaints hotline to identify non-compliant growers. Growers risk losing the use of the *Bt* PIP seed should they be significantly out of compliance in two successive years. The term "grower agreement" refers to any grower purchase contract, license agreement, or similar legal document. In response to reports that compliance with the mandated *Bt* corn PIP refuge requirements was decreasing, each registrant is now required to have an enhanced compliance assurance program (CAP), and a phased requirement for seed bag labeling that clearly shows the refuge requirements (USEPA 2010f). The grower education and compliance assurance program is summarized in Table 11.13.

A technology use guide (TUG) is provided to growers in conjunction with the grower agreement, which stresses the importance of IRM and the need to follow the refuge requirements. Specific information on planting a refuge to comply with

Table 11.13 Summary of grower education and compliance assurance program requirements

Element	Grower education, and compliance assurance program requirements
Grower education program	A program designed to convey to users the importance of complying with the IRM program. The education program involves the use of multiple media, *e.g.*, face-to-face meetings, workshops, written materials such as grower guides, IRM language on bag-tags
Grower agreement	The term "grower agreement" refers to any grower purchase contract, license agreement, or similar legal document. Persons purchasing *Bt* PIP see must sign a grower agreement that specifies the user's legal obligation to follow the IRM requirements. These agreements contractually bind the grower to comply with IRM requirements. Growers should not plant *Bt* PIP corn seed (or *Bt* PIP cotton seed) unless they have signed the Grower/Technology agreement with the company selling the *Bt* PIP product. The signed grower technology agreement is an 'Evergreen' document and must be on file and accessible by the seed company at the time of *Bt* PIP seed purchase by the farmer, but does not need to be signed anew with each bag of seed
Annual grower affirmation	A system which assures that growers will annually affirm that they are contractually bound to comply with the requirements of the IRM program as specified in the grower agreement
Changes to grower agreement	Any changes to grower agreement(s) and any specific stewardship documents should be submitted to EPA at least 30 days before any changes are made
Grower agreement records	Grower agreement records must be kept for three years. They must be available for review by EPA or by a State pesticide regulatory agency that has a mechanism to protect the confidential business information and personally identifiable information contained in the records
Compliance assurance program	A program designed to evaluate the extent to which users purchasing Bt seed are complying with the IRM program and describes actions to address non-compliance using the "phased compliance approach." The program includes: an annual grower survey by an independent third party, annual on-farm assessment program, investigation of legitimate "tips and complaints" that might identify non-compliant users, and a "phased compliance approach" to address non-compliance. Significant non-compliance by an individual user in a two year consecutive period will result in loss of access to the *Bt* seed by that user. The grower survey identifies where there is regional compliance with IRM requirements and assesses grower attitudes toward IRM. Results might be used to increase grower education in a particular region in which there is lower compliance with refuge requirements

the IRM requirements is included in the guide along with diagrams to illustrate acceptable refuge configurations such as separate fields, blocks, and strips. Additional information is provided for insecticide treatments of refuges and other aspects of refuge management. The TUG also describes the circumstances in which a grower may lose access to the *Bt* PIP technology in situations of non-compliance with refuge requirements and information on the compliance assessment monitoring program for refuge planting.

11.23 Evaluation of IRM Strategies: Is Resistance Occurring?

The key to answering the question of whether resistance is occurring is defining what is resistance. The definition of resistance has been adapted and refined over the past 50 years (see summary in Whalon et al. 2008). In 1957, experts from the WHO defined resistance as "the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species" (WHO 1957). In 1986, the National Research Council (NRC 1986) defined resistance as "the inherited ability of a pest strain to tolerate doses of toxicant that would prove lethal to a majority of individuals in a normal population of that species". Similarly, Sawicki (1986) considered resistance to mark "a genetic change in response to selection by toxicants that may impair control in the field." In these later definitions, the distinction was made that resistance development may not economically impair control of the pest in the field. Later, the NRC (1986) definition was adapted to *Bt* toxins and *Bt* crops to describe field-evolved (or field-selected) resistance as a "genetically based decrease in susceptibility of a population to a toxin caused by exposure of the population to the toxin in the field" (Tabashnik et al. 2009). Susceptibility is measured by sampling insects from a field population and determining how their progeny respond to the toxin in laboratory bioassays. Sampling is required annually as part of an approved resistance monitoring program (USEPA 2001). Bioassay results would indicate field-evolved resistance if one or more populations with a history of exposure to the *Bt* toxin in the field are less susceptible to field populations or laboratory strains that have had less exposure (Tabashnik 1994a, b; Tabashnik et al. 2008). A key point is that laboratory documentation of field-evolved resistance may not be an indication of control failures in the field (NRC 1986).

The crop protection industry's Insecticide Resistance Action Committee (IRAC) defines resistance as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (IRAC 2011). Here, resistance must be field-relevant. Reports of field failure might be associated with insect resistance to a *Bt* toxin in the field, but other steps would be needed to "confirm" that repeated field failures were due to a heritable change in the susceptibility of the pest population to the *Bt* toxin, see description of "suspected" and "confirmed" resistance in Sect. 11.20.1 and in the EPA document, "Terms and Conditions for *Bt* Corn Registrations" completed in September 2010 (EPA 2010f).

A number of researchers have demonstrated resistance in the laboratory based on decreased sensitivity when succeeding generations of insect populations are exposed to *Bt* toxins (Ali and Luttrell 2007; Tabashnik et al. 2003a; Bolin et al. 1999; Frutos et al. 1999; Tabashnik 1994; Mahon et al. 2007a; Anilkumar et al. 2008a, b). Resistance monitoring studies conducted in China have indicated increasing Cry1Ac resistance allele frequencies in *H. armigera* (Wu et al. 2006; Wu 2007; Li et al. 2007).

Tabashnik et al. (2008) analyzed more than a decade of resistance monitoring data for six major lepidopteran pests, *H. armigera*, CBW, TBW, ECB, PBW, and *Sesmia nonagrioides,* targeted by *Bt* crops grown in U.S., China, Australia, and Spain. The analysis revealed that Cry1Ac resistance allele frequencies had increased substantially in some populations of CBW collected in Arkansas and Mississippi over the past decade, but resistance allele frequencies had not increased in the five other pests in Australia, China, Spain, and the United States (Tabashnik et al. 2008). Tabashnik et al. (2008) concluded that laboratory bioassays of CBW document the first case of field-evolved resistance to a *Bt* toxin (Cry1Ac) produced by a transgenic crop (Cry1Ac cotton), but stated that CBW resistance to Cry1Ac cotton had not resulted in any field control failures. Moar et al. (2008) published a rebuttal to Tabashnik et al. (2008). The authors argued that significant shifts in resistance allele frequencies are meaningless unless they are linked to field-relevant resistance resulting in control failure.

The challenge is to determine the relationship of field-evolved resistance to field-relevant resistance. Field-evolved resistance to a *Bt* crop may not cause widespread field failure due to a number of factors that affect this relationship including the frequency of resistance alleles, the magnitude of resistance, the extent to which resistance increases the survival in the field (or whether there are fitness costs of resistance), the number and spatial distribution of resistant populations, the insect's population density the availability of alternative control tactics, and the extent to which the insect is a pest (Tabashnik et al. 2009b). For example, Tabashnik et al. (2008) provided the following reasons to explain why field-evolved CBW resistance to Cry1Ac Bollard® cotton did not result in widespread field failures: (1) Not all populations tested were resistant to Cry1Ac, (2) insecticides were always used as an alternative control measure in addition to Bollard® cotton to control CBW, (3) Bollard® cotton caused 48–60% CBW larval mortality in strains 44- to 100-fold resistant to Cry1Ac, and (4) pyramided transgenic cotton producing *Bt* toxins Cry2Ab2 and Cry1Ac (Bollgard II® cotton) introduced in December 2002 will control Cry1Ac-resistant CBW (Tabashnik et al. 2008). In another situation, if a *Bt* crop targets multiple pests then its efficacy would be maintained against pests that maintain their susceptibility to the *Bt* crop even though field-evolved resistance reduces the efficacy to one of the pests (Tabashnik et al. 2009b). The significance of field-evolved resistance to Cry3Bb1 (*Bt* toxin in Cry3Bb1 corn varieties) in some CRW populations in Iowa (Gassmann et al. 2011) to field relevant resistance has yet to be determined.

From 1995 to 2005, there were no documented cases of field resistance (field failures) to *Bt* crops. The first case of field resistance to *Bt* crops was *Spodoptera frugiperda* J. E. Smith (fall armyworm) resistance to Cry1F (TC1507 Herculex® I Insect Protection Maize) maize in Puerto Rico (summarized in Matten et al. 2008 and detailed in Storer et al. 2010). In 2006, unexpected damage reports in Cry1F fields in Puerto Rico caused further investigation as to whether FAW collected in these fields were resistant to the Cry1F protein. Both the screening level and concentration-dependent bioassays on several generations of insects showed that the FAW tested from these fields were greater than 167-fold resistant to the Cry1F toxin expressed in Cry1F maize (Matten et al. 2008; Storer et al. 2010). Cry1F resistance

in FAW populations in Puerto Rico was autosomally inherited and partially recessive (Storer et al. 2010). Resistance was caused by the unique circumstances which increased the selection for FAW resistance in Cry1F corn in Puerto Rico; namely, island geography, high adoption and year round planting of Cry1F corn (Storer et al. 2010). Such circumstances for selection of FAW resistance do not exist in the continental U.S.

A second case of resistance was for the African stem borer, *Busseola fusca* Fuller, to Cry1Ab corn in South Africa. During the 2004–2005 growing season, severe damage to the vegetative stages of Cry1Ab corn hybrids was reported. Field and greenhouse tests documented significant survival and weight gain of the African stem borer larvae collected from the Cry1Ab corn fields in the Vaalharts irrigation scheme compared to non-*Bt* corn areas as evidence of Cry1Ab resistance (van Rensburg 2007). During 2005–2006, additional resistant populations of the African stem borer were collected in another area of the Vaalharts irrigation scheme (Kruger et al. 2011). There was clear evidence that growers ignored the requirement for planting a 20% non-*Bt* maize refuge, as there was 95% adoption of Cry1Ab maize and continuous planting in this region (Kruger et al. 2011).

A third case of resistance is in PBW to Cry1Ac cotton grown in the State of Gujarat in India. India commercialized *Bt* (Cry1Ac) cotton (Bollard® cotton) in 2006, and just 2 years later during the 2008–2009 growing season, there were reports of unexpected PBW survival in several *Bt* cotton fields in Gujarat (Dennehy et al. 2010). Laboratory bioassays confirmed that field survival was associated with major resistance to Cry1Ac (Dennehy et al. 2010; Dhurua and Gujar 2011). This is in sharp contrast to the situation in the U.S. where there have been no confirmed cases of field resistance in the 15 years since Monsanto registered Cry1Ac Bollard® cotton in 1995, the same product registered in India in 2006. While there are requirements for Indian growers to plant a refuge of at least of five rows or a minimum of 20% of the *Bt* cotton field area, it is believed that there was very little compliance with this requirement (Karihaloo and Kumar 2009).

Rapid field resistance to *Bt* crops in these three cases occurred when the requirements of a high dose refuge strategy were not met, along with very high adoption of the technology, continuous planting of the *Bt* crop, and insufficient refuge (Huang et al. 2011). This situation is in striking contrast to that of the U.S. in which there has been wide scale planting of refuges along with the high adoption of *Bt* crops that express a high dose for nearly all of the major pest targets, and no confirmed field resistance. These cases illustrate the importance of a high dose and wide scale adoption of sufficient refuge in delaying the evolution of resistance to *Bt* crops (Tabashnik et al. 2008; Huang et al. 2011).

Most recently, Gassmann et al. (2011) reported that some western CRW populations collected in Iowa had significantly higher survival on Cry3Bb1 corn in laboratory bioassays. In 2009, CRW were collected from Cry3Bb1 corn fields in Iowa where farmers reported severe root damage in three consecutive years and from Cry3Bb1 corn fields which had no reports of unexpected damage. Analysis of the data indicated that there was a positive correlation between higher survival in the laboratory bioassays and collection from severely damaged fields, but no significant

correlation between survival of CRW populations on Cry3Bb1 corn and Cry34/35Ab1 corn (an indication of lack of cross-resistance) (Gassmann et al. 2011). The authors concluded that results from this study indicated field-evolved resistance to Cry3Bb1 in some CRW populations in Iowa (Gassmann et al. 2011). The authors suggested that non-high dose expression in Cry3Bb1 corn and insufficient refuge may have been key factors in the selection for field-evolved resistance (Gassmann et al. 2011). Additional steps would be necessary to "confirm" field-relevant resistance as outlined in Sect. 11.20.2 and described in the *Bt* corn terms and conditions of registration (EPA 2010f).

While it is likely that resistance alleles exist in most pest populations, they are probably rare, transient, local, or unobserved developments. A variety of mitigating factors to resistance development have been suggested including sublethal effects at very small doses in plants, temporal refuge as a result of developmental change or insect behavior (Onstad and Gould 1998b; Gutierrez et al. 2006), predator activity and other population modifying events, the introduction of pyramid products with multiple *Bt* toxins (Sachs et al. 1996; Roush 1998; Stewart et al. 2001; Tabashnik et al. 2002b; Jackson et al. 2003; Zhao et al. 2003, 2005), the presence of biologically active allelochemicals produced by the plants (Olsen and Daly 2000), resistance fitness costs (Carrière et al. 2001b, c, d), and the use of refuges (Tabashnik et al. 2008; Huang et al. 2011).

While some or all of the alternative explanations may be valid, the weight of their contribution in delaying resistance cannot be determined in the absence of field resistance. Some of these factors may be more significant in delaying resistance than others and their contribution, individually or in combination, may have been underestimated or overestimated in insect resistance management simulations. Additional research on the biological, ecological, genetic, and operational factors that influence the evolution of resistance should reduce this uncertainty.

11.24 Summary

In summary, EPA's approach to delaying insect resistance to *Bt* PIPs has been to require an IRM program that is in the public interest in order to maximize the environmental benefits of these products. Such a proactive approach to managing resistance is unique in the world of pesticide regulation (USEPA 2001; Andersen and Matten 2002). EPA's assessment of the risk of insect resistance evolution to *Bt* PIPs is based on the evaluation of genetic, biological, and operational factors as well as simulation modeling. All of the scientific issues concerning IRM for *Bt* PIPs have been scientifically peer-reviewed by EPA's SAP using an open and transparent process. Simulation models have had a significant role in the development and evaluation of IRM strategies and will continue do so in the future. Empirical data are necessary to support the development and use of robust IRM models. As with the use of any model, there are uncertainties that need to be considered when interpreting results. EPA has required that all registered *Bt* PIPs for commercial use have

an IRM program. Each program has the following basic elements: field operational refuge strategy, grower agreements to contractually obligate growers to follow the IRM requirements, resistance monitoring program, remedial action program, grower education program, compliance assurance program, and annual reporting. The refuge strategy, resistance monitoring program, and remedial action programs are based on the specific *Bt* PIP-pest-crop combination. EPA has made changes to IRM requirements for registered *Bt* PIPs in conjunction with new advances in understanding of the evolution of resistance and as novel *Bt* PIP products, which pose different levels of resistance risk, have been registered. Since 1995 when the first *Bt* PIP was registered in the U.S., there have been no cases of confirmed field resistance to *Bt* corn PIPs or *Bt* cotton PIPs grown in the U.S. except the unique situation of FAW resistance to Cry1F corn in Puerto Rico. Wide scale use of required structured refuges has been a contributing factor in delaying the development of insect resistance to *Bt* PIPs (Tabashnik et al. 2008; Huang et al. 2011). Finally, it is important to continue pursuing new scientific information that will improve our understanding of the evolution of insect resistance to *Bt* PIPs and to remain vigilant in the use of IRM strategies to delay the evolution of resistance to *Bt* crops.

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References

Abel CA, Adamczyk JJ Jr (2004) Relative concentration of Cry1a in maize leaves and cotton bolls with diverse chlorophyll content and corresponding larval development of fall armyworm (Lepidoptera: Noctuidae) and southwestern corn borer (Lepidoptera: Crambidae) on maize whorl leaf profiles. J Econ Entomol 97(5):1737–1744

Abney MR, Sorenson CE, Bradley JJR (2004) Alternate crop hosts as resistance management refuges for tobacco budworm in NC. In: Proceedings of the Beltwide Cotton Conferences, San Antonio, Texas. National Cotton Council, pp 1413–1416

Adamczyk JJ Jr, Gore J (2004) Development of bollworms, *Helicoverpa zea*, on two commercial Bollgard® cultivars that differ in overall Cry1Ac levels. J Insect Sci 4:32–36

Adamczyk JJ Jr, Meredith WR Jr (2004) Genetic basis for variability of cry1ac expression among commercial transgenic *Bacillus thuringiensis* (Bt) cotton cultivars in the united states. J Cotton Sci 8:17–23

Adamczyk JJ Jr, Sumerford DV (2001) Potential factors impacting season-long expression of Cry1Ac in 13 commercial varieties of Bollgard® cotton. J Insect Sci 1:13–18

Adamczyk JJ Jr, Hardee DE, Adams LC et al (2001) Correlating differences in larval survival and development of bollworm (Lepidoptera: Noctuidae) and fall armyworm (Lepidoptera: Noctuidae) to differential expression of Cry1A(c) δ-endotoxin in various plant parts among commercial cultivars of transgenic cotton. J Econ Entomol 94(1):284–290

Agi AL, Mahaffey JS, Bradley JR Jr et al (2001) Efficacy of seed mixes of transgenic *bt* and non-transgenic cotton against bollworm, *helicoverpa zea* boddie. J Cotton Sci 5:74–80

Akhurst RJ, James W, Bird LJ, Beard C (2003) Resistance to the Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae). J Econ Entomol 96(4):1290–1299

Ali MI, Luttrell RG (2007) Susceptibility of bollworm and tobacco budworm (Lepidoptera: Noctuidae) to Cry2Ab2 insecticidal protein. J Econ Entomol 100(3):921–931

Ali MI, Luttrell RG, Young SY III (2006) Susceptibilities of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) populations to Cry1Ac insecticidal protein. J Econ Entomol 99(1):164–175

Alphey N, Bonsall MB, Alphey L (2009) Combining pest control and resistance management: synergy of engineered insects with Bt crops. J Econ Entomol 102(2):717–732

Alstad DN, Andow DA (1995) Managing the evolution of insect resistance to transgenic plants. Science 268(5219):1894–1896

Andersen JA, Matten SR (2002) Monitoring case study: insect resistance management in Bt crops (Bt Crop IRM) In: Roseland CR (ed) LMOs and the environment. Proceedings of an international conference held 27–30 November 2001, Raleigh-Durham, NC. Organization for Economic Cooperation and Development, Paris. http://www.oecd.org/dataoecd/40/56/31526579.pdf. Accessed 10 Nov 2011

Andow D (2002) Resisting resistance to Bt-corn. In: Letourneau DK, Burrows BE (eds) Genetically engineered organisms: assessing environmental and human health effects. CRC Press, Boca Raton, pp 99–124

Andow DA, Alstad DN (1998) F_2 screen for rare resistance alleles. J Econ Entomol 91:572–578

Andow DA, Alstad DN, Pang YH, et al (1998) Using an F_2 Screen to Search for Resistance Alleles to *Bacillus thuringiensis* Toxin in European Corn Borer (Lepidoptera: Crambidae) J Econ Entomol 91(3):579–584

Andow DA, Hutchison WD (1998) *Bt* corn resistance management. In: Mellon M, Rissler J (eds) Now or never: serious new plans to save a natural pest control. Union of Concerned Scientists, Cambridge, MA, pp 18–64

Andow D, Ives AR (2002) Monitoring and adaptive resistance management. Ecol Appl 12(5):1378–1390

Andow DA, Olson DM, Hellmich RL et al (2000) Frequency of resistance to *Bacillus thuringiensis* Cry1Ab in an Iowa population of European corn borer (Lepidoptera: Crambidae). J Econ Entomol 93:26–30

Anilkumar KJ, Pusztai M, Moar WJ (2008a) Fitness costs associated with Cry1Ac-resistant *Helicoverpa zea* (Lepidoptera: Noctuidae): a factor countering selection for resistance to Bt cotton? J Econ Entomol 101(4):1421–1431

Anilkumar KJ, Rodrigo-Simon A, Ferré J et al (2008b) Production and characterization of *Bacillus thuringiensis* Cry1Ac-resistant cotton bollworm *Helicoverpa zea* (Boddie). Appl Environ Microbiol 74(2):462–469

Bates SL, Zhao JZ, Roush RT et al (2005) Insect resistance management in GM crops: past, present and future. Nat Biotechnol 23(1):57–62

Berwald D, Matten S, Widawsky D (2006) Economic analysis and regulating pesticide biotechnology at the U.S. Environmental Protection Agency. In: Just RE, Alston J, Zilberman D (eds) Regulating agricultural biotechnology: economics and policy. Springer, New York, pp 21–35

Bird LJ, Akhurst RJ (2004) Relative fitness of cry1a-resistant and –susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on conventional and transgenic cotton. J Econ Entomol 97(5):1699–1709

Bird LJ, Akhurst RJ (2005) Fitness of Cry1A-resistant and –susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on transgenic cotton with reduced levels of Cry1Ac. J Econ Entomol 98(4):1311–1319

Blanco CA, Andow DA, Abel CA et al (2009) *Bacillus thuringiensis* Cry1Ac resistance frequency in tobacco budworm (Lepidoptera Noctuidae). J Econ Entomol 102(1):381–387

Bolin PC, Hutchison WD, Andow DA (1999) Long-term selection for resistance to *Bacillus thuringiensis* Cry1Ac Endotoxin in a Minnesota population of European corn borer (Lepidoptera: Crambidae). J Econ Entomol 92(5):1021–1030

Bourguet D, Genissel A, Raymond M (2000) Insecticide resistance and dominance levels. J Econ Entomol 93(6):1588–1595

Bourguet D, Chaufaux J, Séguin M et al (2003) Frequency of alleles conferring resistance to Bt maize in French and US corn belt populations of the European corn borer, *Ostrinia nubilalis*. Theor Appl Genet 106(7):1225–1233

Bravo A, Gomez I, Conde J et al (2004) Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. Biochim Biophys Acta 1667:38–46

Bravo A, Gill SS, Mario Soberón M (2007) Mode of action of Bacillus thuringiensis Cry and Cyt toxins and their potential for insect control. Toxicon 49(4):423–435

Bravo A, Likitvivatanavong S, Gill SS, et al (2011) *Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem Molec Biol 41(7):423–431

Brookes G, Barfoot P (2008) Global Impact of Biotech Crops: Socio-economic and environmental effects in the first ten years of commercial use. AgBioForum 9(3):139–151

Burd AD, Gould F, Bradley JR et al (2003) Estimated frequency of nonrecessive Bt resistance genes in bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in eastern North Carolina. J Econ Entomol 96(1):137–142

Caccia S, Hernández-Rodríguez CS, Mahon RJ et al (2010) Binding site alteration is responsible for field-isolated resistance to *Bacillus thuringiensis* Cry2a insecticidal proteins in two Helicoverpa species. PLoS One 5(4):e9975. doi:10.1371/journal.pone.0009975

Candas M, Loseva O, Oppert B, et al (2003) Insect resistance to Bacillus thuringiensis, alterations in the Indianmeal moth larval gut proteome. Mol Cell Proteomics 2:19–28

Caprio MA (1998a) Evaluating resistance management strategies for multiple toxins in the presence of external refuges. J Econ Entomol 91(5):1021–1031

Caprio MA (1998b) Insecticide resistance simulation model. http://www.msstate.edu/entomology/Pgjava/ILSImodel.html

Caprio M (2001) Source-sink dynamics between transgenic and non-transgenic habitats and their role in the evolution of resistance. J Econ Entomol 94(3):698–705

Caprio MA, Faver MK, Hankins G (2004) Evaluating the impacts of refuge width on source-sink dynamics between transgenic and non-transgenic cotton. J Insect Sci 4:3. Available online http://www.insectscience.org/4.3

Caprio MA, Storer NP, Sisterson MS et al (2008) Assessing the risk of the evolution of resistance to pesticides using spatially complex simulations models, Chapter 4. In: Whalon ME, Mota-Sanchez D, Hollingworth RM (eds) Global pesticide resistance in athropods. CABI International, Oxfordshire

Carpenter J, Felsot A, Goode T et al (2002) Comparative environmental impacts of biotechnology-derived and traditional soybean, corn, and cotton crops. Council for Agricultural Science and Technology, Ames, Iowa. www.cast-science.org.

Carrière Y, Tabashnik BE (2001) Reversing insect adaptation to transgenic insecticidal plants. Proc Biol Sci 268(1475):1475–1480

Carrière Y, Dennehy TJ, Pedersen B et al (2001a) Large-scale management of insect resistance to transgenic cotton in Arizona: can transgenic insecticidal crops be sustained? J Econ Entomol 94(2):310–325

Carrière Y, Ellers-Kirk C, Liu Y et al (2001b) Fitness costs and maternal effects associated with resistance to transgenic cotton in the pink bollworm (Lepidoptera: Gelechiidae). J Econ Entomol 94(6):1571–1576

Carrière Y, Ellers-Kirk C, Patin A et al (2001c) Overwintering cost associated with resistance to transgenic cotton in the pink bollworm (Lepidoptera: Gelechiidae). J Econ Entomol 94(4):935–941

Carrière Y, Ellers-Kirk C, Pedersen B et al (2001d) Predicting spring moth emergence in the pink bollworm (Lepidoptera: Gelechiidae): implications for managing resistance to transgenic cotton. J Econ Entomol 94(5):1012–1021

Carrière Y, Ellers-Kirk C, Sisterson M et al (2003) Long-term regional suppression of pink bollworm by *Bacillus thuringiensis* cotton. Proc Natl Acad Sci USA 100(4):1519–1523

Carrière Y, Ellers-Kirk C, Biggs R et al (2004a) Effects of gossypol on fitness costs associated with resistance to Bt cotton in pink bollworm. J Econ Entomol 97(5):1710–1718

Carrière Y, Sisterson M, Tabashnik BE (2004b) Resistance management for sustainable use of *Bacillus thuringiensis* crops in integrated pest management. In: Horowitz AR, Ishaaya I (eds) Insect pest management. Springer, Berlin/Heidelberg, pp 65–95

Carrière Y, Ellers-Kirk C, Biggs R et al (2005) Effects of cotton cultivar on fitness costs associated with resistance of pink bollworm (Lepidoptera: Gelechiidae) to Bt cotton. J Econ Entomol 98(3):947–954

Carrière Y, Ellers-Kirk C, Biggs R et al (2006) Cadherin-based resistance to *Bacillus thuringiensis* cotton in hybrid strains of pink bollworm: fitness costs and incomplete resistance. J Econ Entomol 99(6):1925–1935

Cattaneo MG, Yafuso C, Schmidt C et al (2006) Farm-scale evaluation of the impacts of transgenic cotton on biodiversity, pesticide use and yield. Proc Natl Acad Sci USA 103(20):7571–7576

Cerda H, Wright DJ (2004) Modeling the spatial and temporal location of refugia to manage resistance in *Bt* transgenic crops. Agric Ecosyst Environ 102(2):163–174

Chandrashekar K, Kumari A, Kalia V et al (2005) Baseline susceptibility of the American bollworm, *Helicoverpa armigera* (Hüber) to *Bacillus thuringiensis* Berl var. *kurstaki* and its endotoxins in India. Curr Sci 88(1):167–175

Clark TL, Hibbard BE (2004) Comparison of non-maize hosts to support western corn rootworm (Coleoptera: Chrysomelidae) larval biology. Environ Entomol 33(3):681–689

Comins HN (1977) The development of insecticide resistance in the presence of migration. J Theor Biol 64:177–197

Council of Agricultural Science and Technology (CAST) (2004) Management of pest resistance: strategies using crop management, biotechnology, and pesticides. Special Publications – SP24 – June 2004

Crowder DW, Onstad DW (2005) Using generational time-step model to simulate dynamics of adaptation to transgenic corn and crop rotation by Western corn rootworm (Coleoptra: Chrysomelidae). J Econ Entomol 95(2):518–533

Crowder DW, Onstad DW, Gray ME et al (2005) Economic analysis of dynamic management strategies utilizing. Transgenic corn for control of western corn rootworm (Coleoptera: Chrysomelidae). J Econ Entomol 98(3):961–975

Cullen E, DiFonzo C (2011) Handy BT trait table. Michigan State University and University of Wisconsin Extension Services. CDD #028. Updated Oct 2011. http://www.entomology.wisc.edu/cullenlab/extension/xtras/PDFs/Handy_Bt_Trait_Table.pdf. Accessed 24 Oct 2011

Dalecky A, Ponsard S, Bailey RI et al (2006) Resistance evolution to *Bt* crops: predispersal mating of European corn borers. PLoS Biol 4(6):e181. doi:10.1371/journal.pbio.0040181

Davis PM, Onstad DW (2000) Seed mixtures as a resistance management strategy for European corn borers (Lepidoptera: Crambidae) infesting transgenic corn expressing Cry1Ab protein. J Econ Entomol 93(3):937–948

Dennehy TJ, Unnithan GC, Brink SA et al (2004) Update on pink bollworm resistance to Bt cotton in the Southwest (University of Arizona Cooperative Extension, Tucson), Publ. No. P-138; http:__cals.arizona.edu_pubs_crops_az1335_az13357b.pdf

Dennehy TJ, Head GP, Moar W, Greenplate J et al (2010) Status of PBW resistance to Bollgard cotton in India. Abstracts from the 58th ESA meeting 2010, 12–15 Dec 2010

Denolf P, Jansens S, Peferoen, M et al (1993) Two different *Bacillus thuringiensis* delta-endotoxin receptors in the midgut brush border membrane of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae). J Appl Environ Microbiol 59(6):1828–1837

DeRozari MB, Showers WB, Shaw RH (1977) Environment and the sexual activity of the European corn borer. Environ Entomol 6:657–665

Dhurua S, Gujar GT (2011) Field-evolved resistance to *Bt* toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), from India. Pest Manag Sci 67:898–903. doi:10.1002/ps.2127

Dong HA, Li WJ (2007) Variability of endotoxin expression in Bt transgenic cotton. J Agron Crop Sci 193:21–29

Downes S, Mahon R, Olsen K (2007) Monitoring and adaptive resistance management in Australia for Bt-cotton: current status and future challenges. J Invert Pathol 95(3):208–213

Downes S, Parker TL, Mahon RJ (2009) Frequency of alleles conferring resistance to the *Bacillus thuringiensis* toxins Cry1Ac and Cry2Ab in Australian populations of *Helicoverpa punctigera* (Lepidoptera: Noctuidae) from 2002 to 2006. J Econ Entomol 102(2):733–742

Dutton A, D'Alessandro M, Romeis J et al (2004) Assessing expression of *Bt*- toxin (Cry1Ab) in transgenic maize under different environmental conditions. Bull OILB/SROP 27(3):49–55

ERS (Economic Research Service, U.S. Department of Agriculture) (2010) Adoption of genetically engineered crops in the U.S.: extent of adoption. Available at: http://ers.usda.gov/data/biotechcrops/adoption.htm

Fernandez-Cornejo J, Caswell ME (2006) The first decade of genetically engineered crops in the United States. *USDA-ERS Economic Information Bulletin* 11, April 2006 Available at SSRN: http://ssrn.com/abstract=899582

Ferré J, Van Rie J (2002) Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu Rev Entomol 47:501–533

Ferré J, Van Rie J, MacIntosh SC (2008) Insecticidal genetically modified crops and insect resistance management (IRM). In: Romeis J, Shelton AM, Kennedy GG (eds) Integration of insect resistant genetically modified crops within IPM programs. Springer, New York, pp 41–85

Frisvold GB (2006) Bt resistance management: the economics of refuges. In: Just RE, Alston JM, Zilberman D (eds) Regulating agricultural biotechnology: economics and policy. Springer, New York, pp 535–557

Frisvold G, Tronstad R (2002) Economic effects of Bt cotton adoption and the impact of government programs. In: Kalaitsandonakes N (ed) Economic and environmental impacts of agbiotech: a global perspective. Kluwer-Plenum Academic, New York

Frutos R, Rang C, Royer M (1999) Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. Crit Rev Biotechnol 19(3):227–276

Gahan LJ, Gould F, Heckel DG (2001) Identification of a gene associated with *Bt* resistance in *Heliothis virescens*. Science 293(5531):857–859

Gahan LJ, Ma YT, Cobble MLM et al (2005) Genetic basis of resistance to Cry 1Ac and Cry 2Aa in *Heliothis virescens* (Lepidoptera: Noctuidae). J Econ Entomol 98:1357–1368

Gahan LJ, Gould F, Lopez JD Jr et al (2007) A polymerase chain reaction screen of field populations of *Heliothis virescens* for a retrotransposon insertion conferring resistance to *Bacillus thuringiensis* Toxin. J Econ Entomol 100(1):187–194

Gassmann AJ, Carrière Y, Tabashnik BE (2009) Fitness costs of insect resistance to *Bacillus thuringiensis*. Annu Rev Entomol 54:147–163

Gassmann AJ, Petzold-Maxwell JL, Keweshan RS, Dunbar MW (2011) Field-evolved resistance to Bt maize by Western corn rootworm. PLoS One 6(7):e22629. doi:10.1371/journal.pone.0022629

Georghiou GP, Taylor CE (1977) Genetic and biological influences in the evolution of insecticide resistance. J Econ Entomol 70(3):319–323

Glaser JA, Matten SR (2003) Sustainability of insect resistance management strategies for transgenic Bt corn. Biotechnol Adv 22(1–2):45–69

Glaum PR, Ives AR, Andow DA (2012) Contamination and management of resistance evolution to high-dose transgenic insecticidal crops. Theor Ecol 5:195–209

Goldberger J, Merrill J, Hurley T (2005) Bt corn farmer compliance with insect resistance management requirements in Minnesota and Wisconsin. AgBioForum 8(2&3):151–160

Gore J, Leonard BB, Adamczyk JJ (2001) Bollworm (Lepidoptera: Noctuidae) survival on 'Bollgard' and 'Bollgard II' cotton flower bud and flower components. J Econ Entomol 94(6):1445–1451

Gore J, Leonard BR, Church GE et al (2002) Behavior of bollworm (Lepidoptera: Noctuidae) larvae on genetically engineered cotton. J Econ Entomol 95(4):763–769

Gould F (1998) Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu Rev Entomol 43:701–726

Gould F, Tabashnik B (1998) Bt-cotton resistance management. In: Mellon M, Rissler J (eds) Now or never: serious new plans to save a natural pest control. Union of Concerned Scientists, Cambridge, MA, pp 67–105

Gould F, Martinez-Ramirez A, Anderson A et al (1992) Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc Natl Acad Sci USA 89:7986–7990

Gould F, Anderson A, Reynolds A et al (1995) Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J Econ Entomol 88:1545–1559
Gould F, Anderson A, Jones A et al (1997) Initial frequency of alleles for resistance to *Bacillus thuringiensis* toxins in field populations of *Heliothis virescens*. Proc Natl Acad Sci USA 94:3519–3523
Gould F, Blair N, Reid M et al (2002) *Bacillus thuringiensis*-toxin resistance management: Stable isotope assessment of alternate host use by *Helicoverpa zea*. Proc Natl Acad Sci USA 99(26):16581–16586
Gould F, Cohen MB, Bentur JS et al (2006) Impact of small fitness costs on pest adaptation to crop varieties with multiple toxins: a heuristic model. J Econ Entomol 99(6):2091–2099
Greenplate JT (1999) Quantification of *Bacillus thuringiensis* insect control protein cry1ac over time in bollgard cotton fruit and terminals. J Econ Entomol 92(6):1377–1383
Greenplate JT (2004) Report on studies to assess supplemental pyrethroid spray effects on Helicoverpa zea populations in Bollgard® cotton. Monsanto Company, Report number MSL-19252, MRID 46222402. St. Louis, Missouri
Greenplate JT, Mullins JW, Penn SR et al (2003) Partial characterization of cotton plants expressing two toxin proteins from *Bacillus thuringiensis*: relative toxin contribution, toxin interaction, and resistance management. J Appl Entomol 127(6):340–347
Grefenstette B, Osama El-Lissy O, Staten RT (2008) Pink bollworm eradication plan in the U.S. http://www.aphis.usda.gov/plant_health/plant_pest_info/cotton_pests/downloads/pbw-erad-plan2-08.pdf. Updated Feb 2008
Gressel J (2005) Problems in qualifying and quantifying assumptions in plant protection models: resultant simulations can be mistaken by a factor of a million. Crop Prot 24(11):1007–1015
Griffitts JS, Aroian RV (2005) Many roads to resistance: how invertebrates adapt to Bt toxins. Bioessays 27(6):614–624
Griffitts JS, Whitacre JL, Stevens DE, Aroian RV (2001) Bt toxin resistance from loss of a putative carbohydrate-modifying enzyme. Science 293:860–864
Groeters FR, Tabashnik BE, Finson N et al (1994) Fitness costs of resistance to *Bacillus thuringiensis* in the diamondback moth (*Plutella xylostella*). Evolution 48(1):197–201
Gujar GT, Mittal A, Kumari A et al (2004) Host crop influence on the susceptibility of the American bollworm, *Helicoverpa armigera*, to *Bacillus thuringiensis* ssp. kurstaki HD-73. Entomol Exp Appl 113(3):165–172
Gujar GT, Kalia V, Kumari A et al (2007) *Helicoverpa armigera* baseline susceptibility to *Bacillus thuringiensis* Cry toxins and resistance management for Bt cotton in India. J Inverte Pathol 95:214–219
Gunning RV, Dang HT, Kemp FC et al (2005) New resistance mechanism in *Helicoverpa armigera* threatens transgenic crops expressing *Bacillus thuringiensis* Cry1Ac toxin. Appl Environ Microbiol 71(5):2558–2563
Guse CA, Onstad DW, Buschman LL et al (2002) Modeling the development of resistance by stalk-boring Lepidoptera (Crambidae) in areas with irrigated transgenic corn. Environ Entomol 31(4):676–685
Gustafson DI, Head GP, Caprio MA (2006) Modeling the impact of alternative hosts on *Helicoverpa zea* adaptation to bollgard cotton. J Econ Entomol 99(6):2116–2124
Gutierrez AP, Ponsard S (2006) Physiologically based demographics of Bt cotton-pest interactions I. Pink bollworm resistance, refuge and risk. Ecol Model 191(3–4):346–359
Gutierrez AP, Adamczyk JJ, Ponsard S et al (2006) Physiologically based demographics of Bt cotton-pest interactions II. Temporal refuges, natural enemy interactions. Ecol Model 191(3–4):360–382
Hardee DD, Van Duyn JW, Layton MB, Bagwell RD (2001) Bt cotton and management of tobacco budworm and bollworm complex. U.S. Department of Agriculture, Agriculture Research Service, ARS-154. 37pp
Hawkins LS (1986) The role of regulatory agencies in dealing with pesticide resistance. In: Pesticide resistance: strategies and tactics for management. United States National Research

Council's (NRC) Committee on Strategies for the Management of Pesticide Resistant Pest Populations. National Academy Press, Washington, DC, pp 393–403

He K, Wang A, Bai S et al (2006) Efficacy of transgenic Bt cotton for resistance to the Asian corn borer (Lepidoptera: Crambiae). Crop Prot 25:167–173

Head G, Jackson RE, Adamczyk J et al (2010) Spatial and temporal variability in host use by *Helicoverpa zea* as measured by analyses of stable carbon isotope ratios and gossypol residues. J Appl Ecology 47(3):583–592

Heckel DG, Gahan LJ, Gould F et al (1997) Genetics of *Heliothis* and *Helicoverpa* resistance to chemical insecticides and to *Bacillus thuringiensis*. Pestic Sci 51:251–258

Heckel DG, Gahan LJ, Baxter SW et al (2007) The diversity of Bt resistance genes in species of Lepidoptera. J Invertebr Pathol 95(3):192–197

Heimpel GE, Neuhauser C, Andow DA (2005) Natural enemies and the evolution of resistance to transgenic insecticidal crops by pest insects: the role of egg mortality. Environ Entomol 34(3):512–526

Hellmich RL, Pingel R, Hansen WR (1998) Influencing European corn borer (Lepidoptera Crambidae) aggregation sites in small grain crops. Environ Entomol 27:253–259

Hernández CS, Ferré J (2005) Common receptor for *Bacillus thuringiensis* toxins Cry1Ac, Cry1Fa, and Cry1Ja in *Helicoverpa armigera, Helicoverpa zea,* and *Spodoptera exigua*. Appl Environ Microbiol 71(9):5627–5629

Heuberger S, Yafuso C, Degrandi-Hoffman G et al (2008) Outcrossed cottonseed and adventitious *Bt* plants in Arizona refuges. Environ Biosafety Res 7:87–96

Heuberger S, Crowder DW, Brévault T, Tabashnik BE, Carrière Y (2011) Modeling the effects of plant-to-plant gene flow, larval behavior, and refuge size on pest resistance to Bt cotton. Environ Entomol 40(2):484–495. doi:10.1603/EN10247

Hibbard BE, Duran DP, Ellersieck MR et al (2003) Post-establishment movement of western corn rootworm larvae (Coleoptera: Chrysomelidae) in Central Missouri corn. J Econ Entomol 96(3):599–608

Hibbard BE, Higdon ML, Duran DP et al (2004) Role of egg density on establishment and plant-to-plant movement by western corn rootworm larvae (Coleoptera: Chrysomelidae). J Econ Entomol 97(3):871–882

Hibbard BE, Vaughn TT, Oyediran IO et al (2005) Effect of Cry3Bb1expressing transgenic corn on plant-to-plant movement by western corn rootworm larvae (Coleoptera: Chrysomelidae). J Econ Entomol 98(4):1126–1138

Hibbard BE, Clark TL, Ellersieck MR et al (2010) Mortality of western corn rootworm larvae on MIR604 transgenic maize roots: field survivorship has no significant impact on survivorship of F1 progeny on MIR604. J Econ Entomol 103(6):2187–2196. doi:10.1603/EC10179

Hu Y, Andow DA (2011) Field observations of Ostrinia nubilalis eclosion and post-eclosion activity of females around their natal plants. Insect Sci 18(6):712–718

Hua G, Masson L, Jurat-Fuentes JL et al (2001) Binding analyses of *Bacillus thuringiensis* Cry d-Endotoxins using brush border membrane vesicles of *Ostrinia nubilalis*. Appl Environ Microbiol 67(2):872–879

Huang F, Leonard BR, Moore SH et al (2008) Allele frequency of resistance to *Bacillus thuringiensis* Cry1Ab corn in Lousiana populations of sugarcane borer (Lepidoptera: Crambidae). J Econ Entomol 101(2):492–498

Huang F, Parker R, Leonard R et al (2009) Frequency of resistance alleles to Bacillus thuringiensis-corn in Texas populations of the sugarcane borer, *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae). Crop Prot 28(2):174–180

Huang F, Andow DA, Buschman LL (2011) Success of the high-dose/refuge resistance management strategy after 15 years of Bt crop use in North America. Entomol Exp Appl 140:1–16. doi:10.1111/j.1570-7458.2011.01138.x

Hunt TE, Higley LG, Witkowski JF et al (2001) Dispersal of adult European corn borer (Lepidoptera: Crambidae) within and proximal to irrigated and non-irrigated corn. J Econ Entomol 94(6):1369–1377

Hurley TM (2000) Report: a bioeconomic evaluation of the gradual introduction of multiple toxin Bt Corn. prepared for Monsanto

Hurley TM, Babcock BA, Hellmich RL (2001) Bt corn and insect resistance: an economic assessment of refuges. J Agric Resour Econ 26(1):176–194

Hurley TM, Mitchell PD, Rice ME (2004) Risk and the value of Bt corn. Am J Agric Econ 86(2):345–358

Hutchinson WD, Burkness EC, Moon R et al (2007) Evidence of regional suppression of European corn borer populations in transgenic maize in the Midwestern USA: analysis of long-term time series data from three states. In: Proceedings of the XVI international plant protection congress 2007, pp 512–513

Hutchison WD, Burkness EC, Mitchell PD et al (2010) Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science 330:222–225

ILSI (International Life Sciences Institute) (1999) An evaluation of insect resistance management in Bt field corn: a science-based framework for risk assessment and risk management. Report of an expert panel. ILSI Press, Washington, DC. Available online at http://www.ilsi.org/file/h5_IRM.pdf

Insecticide Resistance Action Committee (IRAC) (2011) Definition of resistance. http://www.irac-online.org/about/resistance/. Accessed 18 Nov 2011

Ives AR, Andow DA (2002) Evolution of resistance to Bt crops: directional selection in structured environments. Ecol Lett 5:792–801

Ives AR, Glaum PR, Ziebarth NL et al (2010) The evolution of resistance to two-toxin pyramid transgenic crops. Ecol Appl 21(2):503–515. doi:10.1890/09-1869.1

Jackson RE, Bradley JR Jr, van Duyn JW (2003) Field performance of transgenic cottons expressing one or two *Bacillus thuringiensis* endotoxins against bollworm, *Helicoverpa zea* (Boddie). J Cotton Sci 7(3):57–64

Jackson RE, Bradley JR Jr, Van Duyn JW et al (2004) Comparative production of *Helicoverpa zea* (Lepidoptera: Noctuidae) from transgenic cotton expressing either one or two *Bacillus thuringiensis* proteins with and without insecticide oversprays. J Econ Entomol 97(5):1719–1725

Jackson RE, Bradley JR, Van Duyn JW (2005) Comparative efficacy of Bt technologies against bollworm in North Carolina. In: Proceedings of the Beltwide cotton conference, 4-7 January 2005, New Orleans, LA. National Cotton Council of America, Memphis, TN, pp 1373–1378

Jackson RE, Gould F, Bradley JR Jr et al (2006) Genetic variation for resistance to *Bacillus thuringiensis* toxins in *Helicoverpa zea* (Lepidoptera: Noctuidae) in eastern North Carolina. J Econ Entomol 99(5):1790–1797

Jackson RE, Bradley J, Van Duyn J et al (2008) Regional assessment of Helicoverpa zea populations on cotton and non-cotton crop hosts. Entomol Exp Appl 126:89–106

Johnson KL, Raybould AF, Hudson MD, Poppy GM (2007) How does scientific risk assessment of GM crops fit within the wider risk analysis?, Trends Plant Sci 12(1):1–5, ISSN 1360-1385, 10.1016/j.tplants.2006.11.004

Jurat-Fuentes JL, Adang MJ (2001) Importance of Cry1 d-endotoxin domain II loops for binding specificity in Heliothis virescens (L.). Appl Environ Microbiol 67(1):323–329

Jurat-Fuentes JL, Adang MJ (2006) Cry toxin mode of action in susceptible and resistant *Heliothis virescens* larvae. J Invertebr Pathol 92(3):166–171

Karihaloo JL, Kumar PA (2009) Bt cotton in India – a status report, 2nd edn. Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB), New Delhi, India. p. 56. http://www.apcoab.org/uploads/files/1276753800bt_cotton2.pdf

Kennedy GG, Storer NP (2000) Life systems of polyphagous arthropod pests in temporally unstable cropping systems. Annu Rev Entomol 45:467–493

Knowles BH, Ellar DJ (1987) Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* -endotoxins with different insect specificity. Biochim Biophys Acta 924:507–518

Knox OGG, Gupta VVSR, Nehl DB, Stiller WN (2007) Constitutive expression of Cry proteins in roots and border cells of transgenic cotton. Euphytica 154:83–90

Kranthi KR, Naidu S, Dhawad CS et al (2005) Temporal and intra-plant variability of Cry1Ac expression in Bt-cotton and its influence on the survival of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Noctuidae: Lepidoptera). Curr Sci 89(2):291–298

Kranthi KR, Dhawad CS, Naidu SR et al (2006) Inheritance of resistance in Indian *Helicoverpa armigera* (Hübner) to Cry 1Ac toxin of *Bacillus thuringiensis*. Crop Prot 25(2):119–124

Kranthi S, Dhawad CS, Naidu S et al (2009) Susceptibility of the cotton bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) to the *Bacillus thuringiensis* toxin Cry2Ab before and after the introduction of Bollgard II. Crop Prot 28:371–375

Kruger M, Van Rensburg JRJ, Van Den Berg J (2011) Resistance to Bt Maize in *Busseola fusca* (Lepidoptera: Noctuidae) from Vaalharts, South Africa. Environ Entomol 40(2):477–483. doi:10.1603/EN09220

Langrock I, Hurley TM (2006) Farmer demand for corn rootworm Bt corn: do insect resistance management guidelines really matter? In: Just RE, Alston J, Zilberman D (eds) Regulating agricultural biotechnology: economics and policy. Springer, New York, pp 579–597

Li G-P, Wu K-M, Gould F et al (2007) Increasing tolerance to Cry1Ac cotton from cotton bollworm *Helicoverpa armigera*, was confirmed in Bt cotton farming area of China. Ecol Entomol 32(4):366–375

Linacre NA, Thompson CJ (2003) Dynamics of insect resistance in Bt-corn. Ecol Model 171(3):271–278

Liu YB, Tabashnik BE (1997) Experimental evidence that refuges delay insect adaptation to *Bacillus thuringiensis* toxin Cry1C in diamondback moth. Appl Environ Microbiol 63:2218–2223

Liu YB, Tabashnik BE, Dennehy TJ et al (1999) Development time and resistance to Bt crops. Nature 400(6744):519

Liu YB, Tabashnik BE, Dennehy TJ et al (2001a) Effects of Bt cotton and Cry1Ac toxin on survival and development of pink bollworm (Lepidoptera: Gelechiidae). J Econ Entomol 94(5):1237–1242

Livingston MJ, Carlson GA, Fackler PL (2004) Managing resistance evolution in two pests to two toxins with refugia. Am J Agric Econ 86(1):1–13

Maçon PCRG, Young LJ, Steffey KL et al (1999) Baseline susceptibility of European corn borer (Lepidoptera: Crambidae) to *Bacillus thuringiensis* toxins. J Econ Entomol 92(2):279–285

Mahon RJ, Olsen KM (2009) Limited survival of a Cry2Ab-resistant strain of *Helicoverpa armigera* (Lepidoptera: Noctudae). J Econ Entomol 102(2):708–716

Mahon RJ, Olsen KM, Garsia KA, Young SR (2007a) Resistance to *Bacillus thuringiensis* Toxin Cry2Ab in a Strain of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Australia. J Econ Entomol 100(3):894–902

Mahon RJ, Olsen KM, Downes S, Addison S (2007b) Frequency of alleles conferring resistance to the Bt Toxins Cry1Ac and Cry2Ab in Australian populations of *Helicoverpa armigera* (Lepidoptera: Noctuidae). J Econ Entomol 100(6):1844–1853

Mallet J, Porter P (1992) Preventing insect adaptation to insect-resistant crops: are seed mixtures or refugia the best strategy? Proc R Soc Lond B 250:165–169

Mani GS (1985) Evolution of resistance in the presence of two insecticides. Genetics 109: 761–783

Martins CM, Geyene G, Hofs J-L et al (2008) Effect of water-deficit stress on cotton plants expressing the Bacillus thuringiensis tosin. Ann Appl Biol 152:255–262

Mason CE, Rice ME, Calvin DD, et al (1996) European Corn Borer: Ecology and Management. North Central Regional Publication No. 327. Iowa State University. Ames, Iowa. 57 pp

Matten SR (2006) Technical review of materials supporting Arizona's 24(c) Applications [AZ05009 and AZ050010] to replace structured Non-Bt cotton refuges with sterile insect technology and 100% bollgard and bollgard II cotton (EPA Reg. No. 524–478 and 524–522) to meet their special need for their pink bollworm eradication program [submission dated 3 Aug 2006, MRID#s 469048–00 to −05], 26 Sept 2006. Available at http://www.epa.gov/scipoly/sap/meetings/2006/october/arizonanonbtcottontechreview_2006nofig1-4.pdf

Matten SM, Reynolds AH (2003) Current resistance management requirements for *Bt* cotton in the United States. J New Seeds 5(2–3):137–178

Matten SR, Reynolds AH (2006) BPPD review of data and support materials submitted by Monsanto to amend the insect resistance management requirements for Bollgard II Bt cotton. EPA Reg. No. 524–522. DP Barcode: 327258 Decision: 363974 MRID#: 467172-01, -02, -03. Available at http://www.epa.gov/scipoly/sap/meetings/2006/june/bppd-rev1a.pdf

Matten SR, Hellmich RL, Reynolds AH (2004) Current resistance management strategies for Bt corn in the United States. In: Koul O, Dhaliwal GS (eds) Transgenic crop production: concepts and strategies. Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi, pp 261–288

Matten SR, Head G, Quemada H (2008) How governmental regulation can help or hinder the integration into IPM programs. In: Romeis J, Shelton T, Kennedy G (eds) Integration of insect-resistant genetically modified crops within IPM programs. Springer, New York, pp 27–40

McGaughey WH, Johnson DE (1992) Indianmeal moth (Lepidoptera: Pyralidae) resistance to different stains and mixtures of *Bacillus thuringiensis*. J Econ Entomol 85:1594–1600

Mellon M, Rissler J (eds) (1998) Now or Never: Serious New Plans to Save a Natural Pest Control. Union of Concerned Scientists, Cambridge, MA

Mitchell PD, Hurley TM (2006) Adverse selection, moral hazard, and grower compliance with Bt corn refuge. In: Just RE, Alston JM, Zilberman D (eds) Regulating agricultural biotechnology: economics and policy. Springer, New York, pp 599–623

Mitchell PD, Onstad DW (2005) Effect of extended diapause on evolution of resistance to transgenic *Bacillus thuringiensis* corn by northern corn rootworm (Coleoptera: Chrysomelidae). J Econ Entomol 98(6):2220–2234

Moar WJ, Pusztai-Carey M, Van Faassen H, et al (1995) Development of *Bacillus thuringiensis* CryIC Resistance by *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). Appl Environ Microbiol 61(6):2086–2092

Moar W, Roush R, Shelton A et al (2008) Field-evolved resistance to *Bt* toxins. Nat Biotechnol 26(10):1072–1074

Mohammed-Awel J, Kopecky K, Ringland J (2007) A situation in which a local nontoxic refuge promotes pest resistance to toxic crops. Theor Popul Biol 71(2):131–146

Morin S, Biggs RW, Sisterson MS et al (2003) Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc Natl Acad Sci USA 100(9):5004–5009

NC-205 (North Central Regional Research Committee, NC 205) (1998) Supplement to: Bt Corn & European Corn Borer: Long-Term Success Through Resistance Management, North Central Regional Publication 602. Available at http://www.biotech-info.net/NCR-602_supplement.pdf. October 1998

Nguyen HT, Rehle JA, Rheinpfalz DLR (2007) Quantitative analysis of the seasonal and tissue-specific expression Cry1Ab in transgenic maize MON810. J Plant Dis Prot 114(2):82–87

Nowatzki T, Lefko SA, Binning RR, Thompson SD, Spencer TA, Siegfried BD (2008) Validation of a novel resistance monitoring technique for corn rootworm (Coleoptera: Chrysomelidae) and event DAS-59122-7 maize. J Appl Entomol 132:177–188

NRC (National Research Council) (1986) Pesticide resistance: strategies and tactics for management. National Academy Press, Washington, DC, 486 pp

Olsen KM, Daly JC (2000) Plant-toxin interactions in transgenic Bt cotton and their effect on mortality of *Helicoverpa armigera* (Lepidoptera: Noctuidae). J Econ Entomol 93(4):193–1299

Olsen KM, Daly JC, Holt HE et al (2005) Season-long variation in expression of *Cry1Ac* gene and efficacy of *Bacillus thuringiensis* toxin in transgenic cotton against *Helicoverpa armigera* (Lepidoptera: Noctuidae). J Econ Entomol 98(3):1007–1017

Onstad DW (2006) Modeling larval survival and movement to evaluate seed mixtures of transgenic corn for control of western corn rootworm (Coleoptera: Chrysomelidae). J Econ Entomol 99(4):1407–1414

Onstad DW (2008) Modeling for prediction and management. In: Onstad DW (ed) Insect resistance management: biology, economics and prediction. Academic, London, pp 255–268

Onstad DW, Gould F (1998a) Modeling the dynamics of adaptation to transgenic maize by European corn borer (*Lepidoptera: Pyralidae*). J Econ Entomol 91:585–593

Onstad DW, Gould F (1998b) Do dynamics of crop maturation and herbivorous insect life cycle influence the risk of adaptation to toxins in transgenic host plants? Environ Entomol 27:517–522

Onstad DW, Guse CA, Spencer JL et al (2001) Modeling the dynamics of adaptation to transgenic corn by western corn rootworm (Coleoptera: Chrysomelidae). J Econ Entomol 94:529–540

Onstad DW, Meinke LJ (2010) Modeling evolution of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) to transgenic corn with two insecticidal traits. J Econ Entomol 103(3):849–860

Onstad DW, Guse CA, Porter P et al (2002) Modeling the development of resistance by stalk-boring lepidopteran insects (Crambidae) in areas with transgenic corn and frequent insecticide use. J Econ Entomol 95(5):1033–1043

Onstad DW, Crowder DW, Mitchell PD et al (2003) Economics versus alleles: balancing integrated pest management and insect resistance management for rotation-resistant western corn rootworm (Coleoptera: Chrysomelidae). J Econ Entomol 96(6):1872–1885

Oppert B, Kramer KJ, Johnson D, Upton S, McGaughey WH (1996) Luminal proteinases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* CryIA(c) protoxin. Insect Biochem Mol Biol 26:571–583

Oreskes N (2003) The role of quantitative models in science. In: Canham CE, Cole JL, Lauenroth WK (eds) Models in ecosystem science. Princeton University Press, Princeton, pp 13–31

Orth RG, Head G, Mierkowski M (2007) Determining larval host plant use by a polyphagous lepidopteran through analysis of adult moths for plant secondary metabolites. J Chem Ecol 33:1131–1148

Ostlie KR, Hutchinson WD, Hellmich RL (1997) B.t. corn & European corn borer: long term success through resistance management, North Central Regional Extension Publication NCR 602

Oyediran IO, Hibbard BE, Clark TL (2004) Prairie grasses as alternate hosts of the western corn rootworm (Coleoptera: Chrysomelidae). Environ Entomol 33(3):740–747

Parker CD Jr (2000) Temporal distribution of Heliothines in corn-cotton cropping systems of the Mississippi Delta and relationship to yield and population growth. Ph.D dissertation, Mississippi State University, Mississippi State, Mississippi

Parker CD Jr, Luttrell RG (1999) Interplant movement of *Heliothis virescens* (Lepidoptera: Noctuidae) larvae in pure and mixed plantings of cotton with and without expression of the Cry1Ac δ-Endotoxin protein of *Bacillus thuringiensis* Berliner. J Econ Entomol 92(4):837–845

Peck SL (2000) A tutorial for understanding ecological modeling papers for the nonmodeler. Am Entomol 46(1):40–49

Peck SL, Gould F, Ellner SP (1999) Spread of resistance in spatially extended regions of transgenic cotton: implications for management of *Heliothis virescens* (Lepidoptera: Noctuidae). J Econ Entomol 92:1–16

Pereira EJ, Siqueira HA, Zhuang M et al (2010) Measurements of Cry1F binding and activity of luminal gut proteases in susceptible and Cry1F resistant *Ostrinia nubilalis* larvae (Lepidoptera: Crambidae). J Invertebr Pathol 103:1–7

Pesticide Program Dialogue Committee (PPDC) (1996) Pesticide Program Dialogue Committee Meeting Summary. Available at http://www.epa.gov/oppfead1/cb/ppdc/

Pielke RA Jr (2003) The role of models in prediction for decision. In: Canham CE, Cole JL, Lauenroth WK (eds) Models in ecosystem science. Princeton University Press, Princeton, pp 111–138

Pigott CR, Ellar DJ (2007) Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiol Mol Biol Rev 71(2):255–281

Price JL, Hyde J, Calvin DD (2006) Insect resistance management for Bt corn: an assessment of community refuge schemes. AgBioForum 9(3):129–138

Qureshi JA, Buschman LL, Throne JE et al (2005) Adult dispersal of *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) and its implications for resistance management in Bt-maize. J Appl Entomol 129(6):281–292

Qureshi JA, Buschman LL, Throne JE et al (2006) Dispersal of adult *Diatraea grandiosella* (Lepidoptera: Crambidae) and its implications for corn borer resistance management in *Bacillus thuringiensis* Maize. Annu Entom Soc Am 99(2):279–291

Ravi KC, Mohan KS, Manjunath TM et al (2005) Relative abundance of *Helicoverpa armigera* (Lepidoptera: Noctuidae) on different host crops in India and the role of these crops as natural refuge for *Bacillus thuringiensis* cotton. Environ Entomol 34(1):59–69

Reed JP, Halliday WR (2001) Establishment of Cry9C susceptibility baselines for European corn borer and southwestern corn borer (Lepidoptera: Crambidae). J Econ Entomol 94(2):397–402

Roush RT (1994) Managing Pests and Their Resistance to Bacillus thuringiensis: Can Transgenic Crops be Better Than Sprays? Biocontrol Sci Technol 4:501–516

Roush RT (1997) Bt-transgenic crops: just another pretty insecticide or a chance for a new start in resistance management? Pestic Sci 51:328–334

Roush RT (1998) Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? Philos Trans R Soc Lond B 353(1376): 1777–1786

Ru L, Zhao J, Rui C (2002) A simulation model for adaptation of cotton bollworm to transgenic Bt cotton in northern China. Acta Entomol Sin 45(2):153–159

Sachs ES, Benedict JH, Taylor JF et al (1996) Pyramiding CryIA(b) insecticidal protein and terpenoids in cotton to resist tobacco bud worm (Lepidoptera: Noctuidae). Environ Entomol 25(6):1257–1266

Sachs ES, Benedict JH, Stelly DM et al (1998) Expression and segregation of genes encoding Cry1A insecticidal proteins in cotton. Crop Sci 38:1–11

SAP (FIFRA Scientific Advisory Panel) (1998) Transmittal of the final report of the FIFRA Scientific Advisory Panel meeting held 9–10 Feb 1998, Meeting on scientific issues concerning: *Bacillus thuringiensis* (*Bt*) plant- pesticides and resistance management. (Docket Number: OPPTS-00231). Available online at http://www.epa.gov/scipoly/sap/meetings/1998/february/finalfeb.pdf

SAP (2001) Transmittal of the final report of the FIFRA Scientific Advisory Panel meeting held 18–20 Oct 2000, meeting on scientific issues concerning: *Bt* plant-pesticides risk and benefit assessments. SAP report number 2000–7a. Available online at http://www.epa.gov/scipoly/sap/meetings/2000/october/octoberfinal.pdf

SAP (2002) Transmittal of the final report of the FIFRA Scientific Advisory Panel meeting held 27–29 Aug 2002. Meeting on scientific issues concerning: a rootworm plant-incorporated protectant: non-target insect and insect resistance management issues. Available online at http://www.epa.gov/scipoly/sap/meetings/2002/august/august2002final.pdf 27–29 Aug 2002

SAP (2004) Transmittal of the final report of the FIFRA Scientific Advisory Panel meeting held on 8–10 June 2004, meeting on scientific issues concerning: product characterization, human health risk, ecological risk, and insect resistance management for *Bacillus thuringiensis* (*Bt*) cotton products. SAP Report No. 2004–05

SAP (2006) Transmittal of the final report of the FIFRA Scientific Advisory Panel meeting held 14–15 June 2006, meeting on scientific issues concerning: analysis of a natural refuge of non-cotton hosts for Monsanto's Bollgard II cotton. SAP Minutes No. 2006–03. Available online at http://www.epa.gov/scipoly/sap/meetings/2006/june/june2006finalmeetingminutes.pdf

SAP (2007) Transmittal of the final report of the FIFRA Scientific Advisory Panel meeting held 24–26 Oct 2006, meeting on scientific issues concerning: evaluation of the resistance risks from using 100% Bollgard and Bollgard II cotton as part of a pink bollworm eradication program in the State of Arizona. SAP Minutes No. 2007–01. Available online at http://www.epa.gov/scipoly/sap/meetings/2006/october/october2006finalmeetingminutes.pdf

SAP (2009) Transmittal of the final report of the FIFRA Scientific Advisory Panel meeting held 23–24 Feb 2009, meeting on scientific issues concerning: evaluation of the resistance risks from using a seed mix refuge with Pioneer's Optimum® AcreMax™1 corn rootworm-protected corn. Available online at http://www.epa.gov/scipoly/sap/meetings/2009/february/232009finalreport.pdf

SAP (2011) Transmittal of the final report of the FIFRA Scientific Advisory Panel meeting held 8–9 Dec 2010, meeting on scientific issues concerning: insect resistance management for SmartStax™

Refuge-in-the-Bag, a plant-incorporated protectant (PIP) corn seed blend. Available online at http://www.epa.gov/scipoly/sap/meetings/2010/december/120810minutes.pdf

Sawicki RM (1987) Definition, detection and documentation of insecticide resistance. In: Ford MG, Holloman DW, Khambay BPS, Sawicki RM (eds) Combating resistance to xenobiotics; biological and chemical approaches. Ellis Horwood, Chichester, England, pp 105–117

Sayyed AH, Schuler TH, Wright DJ (2003) Inheritance of resistance to Bt canola in a field-derived population of *Plutella xylostella*. Pest Manag Sci 59(11):1197–1202

Sayyed AH, Gatsi R, Ibiza-Palacios MS et al (2005) Common, but complex, mode of resistance of *Plutella sylostella* to *Bacillus thuringiensis* toxins Cry1Ab and Cry1Ac. Appl Environ Microbiol 71(11):6863–6869

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806

Secchi S, Hurley TM, Babcock BA, Hellmich RL (2006) Managing European corn borer resistance to Bt corn with dynamic refuges. In: Just RE, Alston JM, Zilberman D (eds) Regulating agricultural biotechnology: economics and policy. Springer, New York, pp 559–577

Shelton AM, Zhao J-Z (2008) Resistance management to transgenic plants. In: Radcliffe EB, Hutchinson WD (eds) Integrated pest management. Cambridge University Press, Cambridge

Shelton AM, Tang JD, Roush RT et al (2000) Field tests on managing resistance to Bt-engineered plants. Nat Biotechnol 18(3):339–342

Showers WB, Hellmich RL, Derrick-Robinson ME et al (2001) Aggregation and dispersal behavior of marked and released European corn borer (Lepidoptera: Crambidae) adults. Environ Entomol 30(4):700–710

Siegfried BD, Spencer T, Nearman J (2000) Baseline susceptibility of the corn earworm (Lepidoptera: Noctuidae) to the Cry1Ab toxin from *Bacillus thuringiensis*. J Econ Entomol 93(4):1265–1268

Siegfried BD, Vaughn TT, Spencer T (2005) Baseline susceptibility of western corn rootworm (Coleoptera: Crysomelidae) to Cry3Bb1 *Bacillus thuringiensis* toxin. J Econ Entomol 98(4):1320–1324

Siqueira HAA, Moellenbeck D, Spencer T, Siegfried BD (2004) Cross-resistance of CrylAb-selected *Ostrinia nubilalis* (Lepidoptera: Crambidae) to *Bacillus thuringiensis* δ-endotoxins. J Econ Entomol 97:1049–1057

Sisterson MS, Antilla L, Carrière Y et al (2004) Effects of insect population size on evolution of resistance to transgenic crops. J Econ Entomol 97(4):1413–1424

Sivasupramaniam S, Moar WJ, Ruschke LG et al (2008) Toxicity and characterization of cotton expressing *Bacillus thuringiensis* Cry1Ac and Cry2Ab2 proteins for control of lepidopteran pests. J Econ Entomol 101(2):546–554

Stadelbacher EA (1979) *Geranium dissectum*: an unreported host of the tobacco budworm and bollworm and its role in their seasonal and long term population dynamics in the delta of Mississippi. Environ Entomol 8:1153–1156

Stewart SD, Adamczyk JJ, Knighten KS et al (2001) Impact of Bt cottons expressing one or two insecticidal proteins of *Bacillus thuringiensis* Berliner on growth and survival of Noctuid (Lepidoptera) larvae. J Econ Entomol 94(3):752–760

Stodola TJ, Andow DA, Hyden AR et al (2006) Frequency of resistance to *Bacillus thuringiensis* toxin Cry1Ab in southern United States corn belt population of European corn borer (Lepidoptera: Crambidae). J Econ Entomol 99(2):502–507

Stone TB, Sims SB (1993) Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis*. J Econ Entomol 86:989–994

Storer NP (2003) A spatially explicit model simulating western corn rootworm (Coleoptera: Chrysomelidae) adaption to insect resistant maize. J Econ Entomol 96(5):1530–1547

Storer NP, Babcock JM, Edwards JM (2006) Field measures of western corn rootworm (Coleoptera: Chrysomelidae) mortality caused by Cry34/35Ab1 proteins expressed in maize event 59122 and implications for trait durability. J Econ Entomol 99(4):1381–1387

Storer NP, Babcock JM, Schlenz M, et al (2010) Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* in Puerto Rico. J Econ Entomol 103:1031–1038

Storer NP, Van Duyn JW, Kennedy GG (2001) Life history traits of helicoverpa zea (Lepidoptera: Noctuidae) on non-Bt and Bt transgenic corn hybrids in eastern North Carolina. J Econ Entomol 94(5):1265–1279

Storer NP, Peck SL, Gould F et al (2003a) Spatial processes in the evolution of resistance in *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bt* transgenic corn and cotton in a mixed agroecosystem: a biology-rich stochastic simulation model. J Econ Entomol 96(1):156–172

Storer NP, Peck SL, Gould F et al (2003b) Sensitivity analysis of a spatially-explicit stochastic simulation model of the evolution of resistance in *Helicoverpa zea* (Lepidoptera: Noctuidae) to Bt transgenic corn and cotton. J Econ Entomol 96(1):173–187

Tabashnik BE (1986) Computer simulation as a tool for pesticide resistance management. In: Pesticide resistance: strategies and tactics for management. National Academy Press, Washington, DC, pp 194–206

Tabashnik BE (1994a) Evolution of resistance to *Bacillus thuringiensis*. Annu Rev Entomol 39:47–79

Tabashnik BE (1994b) Delaying insect adaptation to transgenic plants: seed mixtures and refugia-reconsidered. Proc R Soc Lond B Biol 255:7–12

Tabashnik BE, Carrière Y (2007) Evolution of insect resistance to transgenic plants. In: Tilmon KJ (ed) Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects. University of California Press, Berkeley, pp 267–279

Tabashnik BE, Finson N, Groeters FR et al (1994) Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. Proc Natl Acad Sci USA 91:4120–4124

Tabashnik BE, Liu YB, Finson N et al (1997a) One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. Proc Natl Acad Sci USA 94:1640–1644

Tabashnik BE, Liu Y, Malvar T et al (1997b) Global variation in the genetic and biochemical basis of diamondback moth resistance to *Bacillus thuringiensis*. Proc Natl Acad Sci USA 94:12780–12785

Tabashnik BE, Malvar T, Liu YB (1996) Cross-Resistance of the Diamondback Moth Indicates Altered Interactions with Domain II of *Bacillus thuringiensis* Toxins. Appl Environ Microbiol 62(8):2839–2844

Tabashnik BE, Patin AL, Dennehy TJ et al (2000a) Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proc Natl Acad Sci USA 97(24):12980–12984

Tabashnik BE, Liu YB, de Maagd RA, Dennehy TJ (2000b) Cross-resistance of pink bollworm (*Pectinophora gossypiell*a) to *Bacillus thuringiensis* toxins. Appl Environ Microbiol 66:4582–4584

Tabashnik BE, Liu YB, Dennehy TJ et al (2002a) Inheritance of resistance to Bt toxin Cry1Ac in a field-derived strain of pink bollworm (Lepidoptera: Gelechiidae). J Econ Entomol 95(5): 1018–1026

Tabashnik BE, Dennehy TJ, Sims MA et al (2002b) Control of resistant pink bollworm (*Pectinophora gossypiella*) by transgenic cotton that produces *Bacillus thuringiensis* toxin Cry2Ab. Appl Environ Microbiol 68(8):3790–3794

Tabashnik BE, Carrière Y, Dennehy TJ et al (2003a) Insect resistance to transgenic *Bt* Crops: lessons from the laboratory and field. J Econ Entomol 96(4):1031–1038

Tabashnik BE, Dennehy TJ, Carrière Y et al (2003b) Resistance management: slowing pest adaptation to transgenic crops. Acta Agric Scand B 53(Suppl 1):51–56

Tabashnik BE, Gould F, Carrière Y (2004) Delaying evolution of insect resistance to transgenic crops by decreasing dominance and heritability. J Evol Biol 17:904–912

Tabashnik BE, Biggs RW, Higginson DM et al (2005a) Association between resistance to Bt cotton and cadherin genotype in pink bollworm. J Econ Entomol 98(3):635–644

Tabashnik BE, Dennehy TJ, Carrière Y (2005b) Delayed resistance to transgenic cotton in pink bollworm. Proc Natl Acad Sci USA 102(43):15389–15393

Tabashnik BE, Fabrick JA, Henderson S et al (2006) DNA screening reveals pink bollworm resistance to Bt cotton remains rare after a decade of exposure. J Econ Entomol 99(5):1525–1530

Tabashnik BE, Gassmann AJ, Crowder DW et al (2008) Insect resistance to Bt crops: evidence versus theory. Nat Biotechnol 26(2):199–202

Tabashnik BE, Unnithan GC, Masson L et al (2009a) Asymmetrical cross-resistance between *Bacillus thuringiensis* toxins Cry1Ac and Cry2A in pink bollworm. Proc Natl Acad Sci USA 106:11889–11984

Tabashnik BE, Van Rensburg JBJ, Carriere Y (2009b) Field-evolved insect resistance to *Bt* crops: definition, theory, and data. J Econ Entomol 102(6):2011–2025

Tabashnik BE, Huang F, Ghimire MN et al (2011) Efficacy of genetically modified Bt toxins against insects with different genetic mechanisms of resistance. Nat Biotechnol. doi:10.1038/nbt.1988, Published online 9 Oct 2011

Tang JD, Collins HL, Metz TD et al (2001) Greenhouse tests on resistance management of Bt transgenic plants using refuge strategies. J Econ Entomol 94:240–247

Tate CD, Hellmich RL, Lewis LC (2006) Evaluation of *Ostrinia nubilalis* (Lepidoptera: Crambidae) neonate preferences for corn and weeds in corn. J Econ Entomol 99(6):1987–1993

Taylor CE (1983) Evolution of resistance to insecticides: the role of mathematical models and computer simulations. In: Georghiou BP, Saito T (eds) Pest resistance to pesticides. Plenum Press, New York, pp 163–173

Thompson GD, Head G (2001) Implication of regulating insect resistance management. Am Entomol (Spring) 47:6–10

Thompson GD, Matten S, Denholm I et al (2008) The politics of resistance management: working towards pesticide resistance management globally. In: Whalon ME, Mota-Sanchez D, Hollingworth RM (eds) Global pesticide resistance in arthropods. CAB International, Wallingford, pp 146–165

Torres JB, Ruberson JR Jr (2006) Spatial and temporal dynamics of oviposition behavior of bollworm and three of its predators in Bt and non-Bt cotton fields. Entomol Exp Appl 120(1):11–22

Trisyono YA, Chippendale GM (2002) Susceptibility of field-collected populations of the Southwestern corn borer, *Diatraea grandiosella*, to *Bacillus thuringiensis*. Pest Manag Sci 58:1022–1028

U.S. Environmental Protection Agency (1998) The Environmental Protection Agency's white paper on Bt plant-pesticide resistance management. Biopesticides and Pollution Prevention Division, Washington, DC; EPA/739/S-98/001. Available online at http://www.epa.gov/EPA-PEST/1998/January/Day-14/paper.pdf

U.S. Environmental Protection Agency (1999) EPA and USDA position paper on insect resistance management in *Bt* crops May 27, 1999 (minor revisions December 7, 1999). Available at: http://www.epa.gov/pesticides/biopesticides/otherdocs/bt_position_paper_618.htm

U.S. Environmental Protection Agency (2000) Response of the Environmental Protection Agency to petition for rulemaking and collateral relief concerning the registration and use of genetically engineered plants expressing *Bacillus thuringiensis* endotoxins, submitted by petitioners Greenpeace International, International Federation of Organic Agriculture Movements, International Center For Technology Assessment, et al. April 19, 2000. Available at: http://www.epa.gov/opp00001/biopesticides/pips/greenpeace-petition.pdf

U.S. Environmental Protection Agency (2001) Biopesticides registration action document: *Bacillus thuringiensis* plant-incorporated protectants (16 Oct 2001). Biopesticides and Pollution Prevention Division, Washington, DC. Available online at http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm

U.S. Environmental Protection Agency (2010a). Biopesticides registration action document: Cry1Ab and Cry1F *Bt* plant-incorporated protectants, 10 Sept 2010. Available at http://www.epa.gov/oppbppd1/biopesticides/pips/cry1f-cry1ab-brad.pdf

U.S. Environmental Protection Agency (2010b) Biopesticides registration action document: *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (PHP17662 T-DNA) in Event DAS-59122-7 Corn (OECD Unique Identifier: DAS-59122-7), 10 Sept 2010. Available at http://www.epa.gov/oppbppd1/biopesticides/pips/cry3435ab1-brad.pdf

U.S. Environmental Protection Agency (2010c) Biopesticides registration action document: *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production (Vector PV-ZMIR13L) in MON 863 Corn (OECD Unique Identifier: MON-ØØ863-5) and *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production (Vector PV-ZMIR39) in MON 88017 Corn (OECD Unique Identifier: MON-88Ø17-3). Available at http://www.epa.gov/oppbppd1/biopesticides/pips/cry3bb1-brad.pdf

U.S. Environmental Protection Agency (2010d) Biopesticides registration action document: modified Cry3A protein and the genetic material necessary for its production (Via Elements of pZM26) in Event MIR604 Corn SYN-IR604-8, 10 Sept 2010. Available at http://www.epa.gov/oppbppd1/biopesticides/pips/mcry3a-brad.pdf.fs

U.S. Environmental Protection Agency (2010e) Biopesticides registration action document: *Bacillus thuringiensis* Cry1A.105 and Cry2Ab2 insecticidal proteins and the genetic material necessary for their production in corn, 10 Sept 2010. Available at http://www.epa.gov/oppbppd1/biopesticides/pips/mon-89034-brad.pdf

U.S. Environmental Protection Agency (2010f) Terms and conditions for Bt corn registrations 30 Sept 2010. Office of Pesticide Programs, Washington, DC. Available online at http://www.epa.gov/oppbppd1/biopesticides/pips/bt-corn-terms-conditions.pdf

U.S. Environmental Protection Agency (2011) Insect Resistance Management Fact Sheet for *Bacillus thuringiensis (Bt)* Cotton Products. Available online at: http://www.epa.gov/oppbppd1/biopesticides/pips/bt_cotton_refuge_2006.htm, Updated February 16, 2011.

U.S. Environmental Protection Agency (EPA), Office of the Science Advisor, Council for Regulatory Environmental Modeling (2009) Guidance on the development, evaluation, and application of regulatory environmental models. U.S. EPA Council for Regulatory Environmental Modeling. EPA/100/K-09/003 March 2009, Available online at http://www.epa.gov/crem/library/cred_guidance_0309.pdf

Vacher D, Bourguet D, Rousset F et al (2003) Modelling the spatial configuration of refuges for a sustainable control of pests: a case study of Bt cotton. J Evol Biol 16:378–387

Vacher D, Bourguet D, Rousset F et al (2004) High dose refuge strategies and genetically modified crops – reply to Tabashnik et al. J Evol Biol 17(4):913–918

Vacher D, Bourguet D, Desquilbet M et al (2006) Fees or refuges: which is better for the sustainable management of insect resistance to transgenic Bt corn. Biol Lett 2:198–202

van Rensburg JBJ (2007) First report of field resistance by the stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. S Afr J Plant Soil 24(3):147–151

Venette RC, Hutchison WD, Andow DA (2000) An In-Field Screen for Early Detection and Monitoring of Insect Resistance to Bacillus thuringiensis in Transgenic Crops. J Econ Entomol 93(4):1055–1064

Walker K, Mendelsohn M, Matten S et al (2003) The role of microbial Bt products in US crop protection. In: Metz M (ed) *Bacillus thuringiensis*: a cornerstone of modern agriculture. Food Products Press, Binghamton, pp 31–51

Wan P, Zhang Y, Wu K et al (2005) Seasonal expression profiles of insecticidal protein and control efficacy against *Helicoverpa armigera* for Bt cotton in the Yangtze River Valley of China. J Econ Entomol 98(1):195–201

Wenes A-L, Bourguet D, Andow DA et al (2006) Frequency and fitness cost of resistance to *Bacillus thuringiensis* in *Chrysomela tremulae* (Coleoptera: Chrysomelidae). Heredity 97:127–134

Whalon ME, Mota-Sanchez D, Hollingworth RM (2008) Analysis of global pesticide resistance. In: Whalon ME, Mota-Sanchez D, Hollingworth RM (eds) Global pesticide resistance in arthropods. CAB International, Wallingford, pp 5–31

Wilson TA, Hibbard BE (2004) Host suitability of nonmaize agroecosystem grasses for the western corn rootworm (Coleoptera: Chrysomelidae). Environ Entomol 33(4):1102–1108

World Health Organization (WHO) (1957) Expert committee on malaria, seventh report. WHO technical report series No. 125. World Health Organization, Geneva

Wu K (2007) Monitoring and management strategy for *Helicoverpa armigera* resistance to Bt cotton in China. J Invertebr Pathol 95(3):220–223

Wu KM, Guo YY (2005) The evolution of cotton pest management practices in China. Annu Rev Entomol 50:31–52

Wu K, Guo Y, Head G (2006) Resistance Monitoring of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to Bt Insecticidal Protein During 2001-2004 in China. J Econ Entomol 99(3): 893–898

Wu K, Goa S, Guo Y (2002) Evaluation of the natural refuge function for *Helicoverpa armigera* (Lepidoptera: Noctuidae) within *Bacillus thuringiensis* transgenic cotton growing areas in north China. J Econ Entomol 95(4):832–837

Wu K, Guo Y, Lu N et al (2003) Efficacy of transgenic cotton containing a cry1Ac gene from *Bacillus thuringiensis* against *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Northern China. J Econ Entomol 96(4):1322–1328

Wu KM, Lu YH, Feng HQ et al (2008) Suppression of cotton bollworm in multiple crops in China in areas with Bt toxin-containing cotton. Science 321:1676–1678

Xu X, Yu L, Wu Y (2005) Disruption of a cadherin gene associated with resistance to Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. Appl Environ Microbiol 71(2):945–954

Zhang X, Candas M, Griko NB, Taussig R, Bulla LA Jr (2006) A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. Proc Natl Acad Sci USA 103:9897–9902

Zhao J-Z, Cao J, Li Y et al (2003) Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat Biotechnol 21(12):1493–1497

Zhao J, Cao J, Collins HC et al (2005) Concurrent use of transgenic plants expressing a single and two Bt genes speed insect adaptation to pyramided plants. Proc Natl Acad Sci USA 102:8426–8430

Chapter 12
Development and Regulation of the *Plum Pox Virus* Resistant Transgenic Plum 'HoneySweet'

Ralph Scorza, Ann Callahan, Michel Ravelonandro, and Michael Braverman

Abstract Genetic engineering (GE) can target specific genetic improvements and allow for the development of novel, useful traits. In spite of the potential utility of GE for fruit tree improvement, the technology has not, to date, been widely exploited for variety development due, in part, to the reticence of researchers to become involved in the regulatory process. Over the past 20 years an intensive international research project focused on the development of GE resistance to *Plum pox virus* (PPV) the causative agent of Sharka, one of the most destructive diseases of plum and other stone fruits. This effort resulted in the development of 'HoneySweet' plum, a GE variety that has proven to be highly resistant to PPV, as demonstrated in over 15 years of field testing in the U.S. and Europe. In order to make this variety available to breeders and growers in the U.S., dossiers were submitted to the U.S. regulatory agencies. This process ultimately led to the regulatory approval of 'HoneySweet' in the U.S. The work with 'HoneySweet' demonstrates that the regulatory process, while a significant effort, can be successfully navigated by public institution researchers. Nevertheless, the few examples of such success demonstrate a need for public institutions to find ways to encourage, support and reward researchers who pursue deregulation efforts. The long-standing successes of virus control in

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squash and papaya, and the current work with plum demonstrate the power and the safety of GE for specialty crop improvement. The commitment of researchers, institutional support, clear, science-based regulatory frameworks that build upon a developing knowledge base, industry support, and public outreach are components that are now necessary to move this technology forward to improve agricultural production and its sustainability.

Keywords *Prunus* • Plum • *Plum Pox Virus* • Virus resistance • Genetic engineering • Sharka • Gene silencing • Rosaceae • GE regulations • APHIS • EPA • FDA

12.1 Introduction

Genetically engineered (GE) genotypes now account for the greater part of the world acreage of some of the most widely grown and traded crops such as soybean, maize, cotton, and canola. In the West, the research and development of these GE crop varieties have been virtually the exclusive domain of large multinational corporations. These corporations have the financial resources not only to run extensive molecular research programs and breeding trials but they can also heavily invest in intellectual property (IP) issues and most importantly, they can invest the significant resources necessary for regulatory approvals, in most cases in multiple countries. The payback on these investments comes from crops with significant world-wide production. Typically, specialty crops are high value per unit land area but they are produced on relatively small land areas and are made up of a multitude of genotypes specific to particular regions and/or markets. If the production of GE varieties of specialty crops is to move forward it will likely be through the work of public institutions. The need for the use of GE technologies for the improvement of specialty crops is great. As a whole, these crops produce high incomes for growers, contribute significantly to local and regional economies, and are important components of a healthy diet. But public institutions suffer from limited funding, and industries for each specialty crop are relatively small and so cannot provide the funding necessary for robust programs that will take a GE crop variety from proof of concept to product. Public research institutions also suffer from limited experience and limited staff that can be devoted to IP and regulatory issues. University researchers are not rewarded for time spent on IP and regulatory work but instead are awarded tenure and grants for novel research that then may be taken to the stage of proof-of-concept. Research in model plants demonstrating the expression of novel transgenes with potential for crop improvement generally ends with publication but without a commercial product. Such findings may be the starting point for private enterprise to enter, taking the proof-of-concept to product, as seen with the major row crops. Unfortunately, this has generally not occurred with specialty crops for several reasons, including freedom to operate issues, and the time, costs, and uncertainties associated with regulatory approvals. The uncertainty of consumer acceptance also figures largely in the decision process of private enterprise.

The difficulties encountered in the path from proof-of-concept to GE specialty crop marketing are significant and they are real. World-wide there are only nine specialty crops in which a GE variety has been marketed or taken to the point where it can be marketed; these are tomato, potato, squash, sweet corn, papaya, flax, tobacco, carnation, and plum. This chapter will focus on the development, testing, and regulatory approval of 'HoneySweet' plum, genetically engineered for resistance to Plum pox virus (PPV), to illustrate the path from research to product taken by a public institution, the United States Department of Agriculture (USDA), Agricultural Research Service (ARS).

12.2 Background for the GE Approach

Sharka disease caused by Plum pox virus (PPV) is considered to be one of the most serious threats to stone fruit production world-wide (Cambra et al. 2006). Symptoms include fruit deformation, pitting and gumming of fruit flesh, premature fruit drop, leaf chlorosis, and in highly susceptible varieties, tree decline. Almost all species of the genus *Prunus* are susceptible (Damsteegt et al. 2006). Since its first description in Bulgaria (Atanassov 1932), the virus has spread to a large part of the European continent, around the Mediterranean basin and Near and Middle East, South and North America (Argentina, Canada, Chile, and USA) and Asia (China, Kazakhstan and Pakistan) (Cambra et al. 2006; various authors 2006) (Fig. 12.1). Long distance dispersion of the virus is through infected budwood and rootstocks. Local spread is by aphids. In order to restrict the spread of PPV the European Plant Pathology Organization (EPPO) recommends measures such as quarantine isolation, nursery

Fig. 12.1 Spread of Plum pox virus following its identification in Europe (Bulgaria) in 1918 (Atanassov 1932)

and orchard surveys, propagation of virus-free *Prunus* and chemical treatment of trees against aphid vectors. These measures have been ineffective in halting the spread of PPV which is now endemic in many European countries. Due to the rapid spread of PPV by aphids and the presence of many potential hosts, Sharka disease is difficult to eradicate once it has become established. Tree removal is the only strategy that can be used to eradicate the virus from an area. While the control of PPV through host resistance represents a preferred strategy there are few sources of high level resistance and therefore stonefruits, in general, are highly vulnerable.

12.3 Research Approach

Facing the threat of the introduction of PPV into the U.S., USDA-ARS began a program of pre-emptive breeding for PPV resistance. In 1989 researchers at the ARS- Appalachian Fruit Research Station (AFRS), Kearneysville, West Virginia began work on the development of resistance to PPV through genetic engineering. Our first studies utilized the papaya ringspot virus (PRV) coat protein (CP) gene (kindly provided by Dr. Dennis Gonsalves, Cornell University, Geneva, NY; currently USDA-ARS, Hilo, HI) which was used to develop PRV resistant papayas (Gonsalves 1998). It was thought that this virus CP gene might have enough homology to the PPV-CP gene to be effective in providing resistance to PPV. At the time that this work began, virus resistance was expected to be CP-mediated (Beachy et al. 1990). The heterologous protection against PPV in plum based on PRV-CP expression was effective for several years in greenhouse tests, but after 32 months symptoms of PPV infection appeared and plants became fully infected (Scorza et al. 1995). During the time of this work in the U.S., Michel Ravelonandro (INRA, Bordeaux, France) had isolated, sequenced and cloned the PPV coat protein (CP) gene (Ravelonandro et al. 1992). In collaboration with Ravelonandro, Gonsalves, and members of Gonsalves' research group, the PPV-CP gene was engineered into the plasmid pGA482GG (Fitch et al. 1990; Ling et al. 1991), the same plasmid that was used for the successful engineering of papaya ringspot virus resistant papayas (Fitch et al. 1992). *Agrobacterium*-mediated transformation of plum was based on the procedure developed by Mante et al. (1991) utilizing hypocotyl slices from seed derived from open pollination. The first 2 years of the project were dedicated to vector construction and testing in tobacco, transformation of plum, tissue culture of putative GE plants (selection, proliferation, rooting), greenhouse acclimation and plant propagation for testing. Confirmed transgenic plants were transferred under a USDA-Animal and Plant Health Inspection Service (APHIS) permit to the BSL3-P containment greenhouse at the USDA-ARS Foreign Disease and Weed Research Unit at Ft. Detrick, MD. At that time it was the only greenhouse facility in the U.S. where work with PPV was permitted. During the 3 years of these greenhouse-based inoculation and testing studies, one transgenic plum line appeared to be highly resistant to PPV. However, this line did not express PPV-CP and produced barely detectable levels of CP mRNA. Clones that did express the CP gene proved to be

susceptible (Ravelonandro et al. 1997; Scorza et al. 2001). This suggested that a mechanism other than CP-mediated protection was at work. The "C5" plum clone became the focus of research on the mechanism of resistance to PPV. From these studies, a series of papers describing the resistance in the greenhouse and field led to the demonstration of post-transcriptional gene silencing (PTGS) as the mechanism of resistance (Ravelonandro et al. 1997; Scorza et al. 2001; Hily et al. 2004, 2005). Silencing was based on the activity of a hairpin configuration that was apparently the result of a duplication and rearrangement during the insertion event. In 1993, a field trial of C5 and the other transgenic lines was planted at the AFRS in Kearneysville, WV under an APHIS permit. This field trial was developed not to test for resistance, since PPV was not present in the U.S. and we could not inoculate plants in the field, but rather to evaluate the trees for transgene expression and for their horticultural traits including growth habit, and fruit yield and quality. While the C5 clone appeared to be highly resistant in greenhouse tests, field testing under artificial inoculation and natural aphid-vectored disease pressure was necessary to evaluate resistance on mature trees under typical orchard conditions and in different plum-growing environments, and with different PPV strains. Collaborations were developed with research partners in Europe (T. Malinowski, Poland; I. Zagrai, Romania; and M. Cambra, Spain) to test this resistant clone in areas where PPV was established. Appropriate field test permits were granted in each country and field trials were initiated in 1996–1997, which was 6–7 years following the initial plum transformations. By 2002 the field tests clearly demonstrated the resistance of C5 to PPV infection through aphid vectors and by graft inoculation (Hily et al. 2004). Continuation of these tests through 2005 confirmed the resistance (Malinowski et al. 2006).

In December 1999, PPV was detected in peach and plum trees in orchards in Adams County, Pennsylvania (Levy et al. 2000). This detection resulted in what was to become a 10-year eradication program that cost over $65 M and resulted in almost the complete elimination of stone fruits in the affected counties. At that same time 'HoneySweet', the variety name for C5, was demonstrating an extremely high level of resistance to PPV in the European field trials. C5 trees exposed to natural aphid vectors were never found to be infected, and graft-inoculated trees showed only low virus titer near the point of graft inoculation. With the detection of PPV in the U.S., the need for resistant germplasm for U.S. growers was clear and it was decided to make 'HoneySweet' available for U.S. breeders and growers.

12.4 The Regulatory Process

The commercial availability of 'HoneySweet' required regulatory approvals from APHIS, and the U.S. Environmental Protection Agency (EPA). A voluntary submission to the U.S. Food and Drug Administration (FDA) is also typically a part of the regulatory process for GE food products. With the anticipation of regulatory submissions, risk assessment studies were initiated both in the U.S. and in Europe.

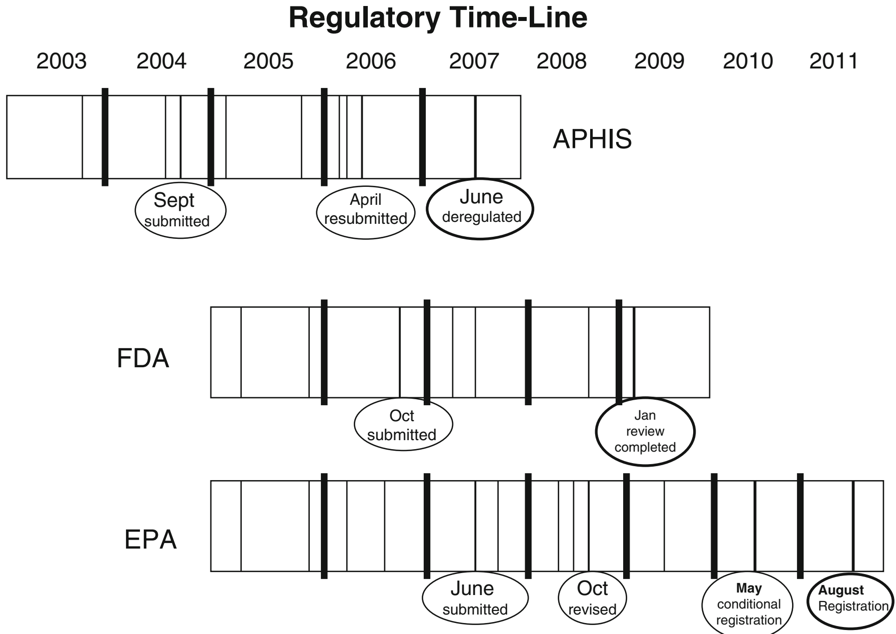

Fig. 12.2 Schedule of regulatory consultations (*thin lines*), submissions and approvals for 'HoneySweet' plum. Thin vertical lines indicate dates of meetings between regulators and applicant

Pre-submission consultations with U.S. regulatory agencies APHIS, FDA and EPA began in 2003 (Fig. 12.2). APHIS has jurisdiction over the field testing of genetically engineered plants that contain plant pathogen genes or promoters. FDA has jurisdiction over GE plants used as food, and EPA regulates GE crop plantings of over ten acres for GE plants that produce molecules that protect plants against pests - protection against PPV in the case of 'HoneySweet'. Based upon the guidance provided in these consultations, data from over 13 years of work with 'HoneySweet' in the laboratory, greenhouse and field, in the U.S. and in Europe, including risk assessment studies, were incorporated into dossiers for the regulatory agencies. An application for determination of non-regulatory status was submitted to APHIS in September 20, 2004. In February 2005 a notification from APHIS was received detailing deficiencies and clarifications that needed to be addressed in the application. The revised application was resubmitted on March 13, 2006 and deemed to be complete and accepted for review on April 7, 2006. At that time APHIS initiated, as part of its standard procedure, an Environmental Assessment (EA). In May, 2006, the petition submitted to APHIS was posted on the internet for 60 days of public comment. APHIS received 1,725 comments, 1,708 were not in support of deregulation. Many if not most comments of non-support appeared to be duplicates, cut and pasted from a single anti-GMO website. APHIS addressed the comments and a

determination of non regulated status was made on June 27, 2007. The result of the EA was a Finding of No Significant Impact (FONSI).

The dossier provided to the FDA consisted of information pertaining to the food uses of plum, and compositional analyses of 'HoneySweet' and control, untransformed plums. To obtain this data fruit samples from several varieties of plum of similar age and located near the 'HoneySweet' planting were collected and sent to a commercial laboratory for analysis. Information pertaining to allergenicity and antinutrients was obtained through the collaboration of ARS colleagues at the USDA-ARS- Eastern Regional Research Center, Wyndmoor, PA. The purpose of the analyses was to determine if any transgene sequences would be predicted to produce proteins that matched known allergenic or anti-nutrient proteins. Several databases and alignment approaches were used including the Allermatch allergen finder (www.allermatch.org), 7 and 8 amino acid word search using the same database, 80 amino acids sliding window alignment with the same database, and FASTA alignments done manually using the Codex Alimentarius guidelines which were used to create the Allermatch algorithms. The sequence was broken into 80 amino acid words and FASTA aligned with allergens (http://www.who.int/foodsafety/publications/biotech/en/ec_jan2001.pdf).

The antinutrient potential of the insert sequences was evaluated using the NCBI antinutirent sequence data base. The submission to FDA was made on October 26, 2006 and was accepted on January 12, 2007. Additional information and\or clarifications were provided at the request of FDA on April 5, June 3, June 12, 2007 and on September 19, 2008. A final letter of "no further questions" was received from FDA on January 16, 2009. In the language typical of such a letter, the FDA stated that, "Based on the safety and nutritional assessment USDA-ARS conducted, it is the understanding of FDA that USDA-ARS has concluded that plums derived from the new variety are not materially different in composition, safety, and other relevant parameters from plums currently on the market and that the genetically engineered plum line C5 does not raise issues that would require premarket review of, or approval by, FDA."

Although 'HoneySweet' produced no PPV-CP and although PPV-infected plums – which are widely consumed in Europe-- contain PPV-CP, EPA determined that the PPV-CP gene in 'HoneySweet' plum would be considered as a plant incorporated protectant (PIP) and that 'HoneySweet' should be regulated and registered as a biopesticide. The format for EPA registration of a biopesticide is administratively complex. In order to expedite the submission process and allow the researchers to focus on putting together the necessary scientific documentation rather than working on the administrative issues of the EPA regulatory process, ARS sought the assistance of the Interregional Research Project Number 4 (IR-4), an organization that functions to submit minor use pesticide registration packages and tolerance petition applications to EPA. IR-4 assumed the responsibility of taking the data provided by ARS researchers and developing a submission package that conformed to the formatting requirements of EPA. The dossier was submitted in June, 2007. The submission included a Registration Volume of administrative materials and four additional volumes consisting of Volume 1- Tolerance Exemption petition for the PPV resistance gene (the PPV-CP gene); Volume 2, Product Chemistry of the PPV

Resistance Gene; Volume 3 PPV - Resistance Gene Non-target Waiver Requests; and Volume 4 - PPV Resistance Gene Health Waiver Requests. The submission was found to be in compliance with the data submission standards contained in Pesticide Registration (PR) Notice 86-5 (see http://www.epa.gov/PR_Notices/pr86-5.html). During the review period EPA made several requests to the ARS submitter for conformance to EPA documentation guidelines and clarification of information and submission of additional information. Each request "stopped the clock" on the review process, adding additional time to the EPA review process. The initial scientific review resulted in a September 2007 request for additional information. This request required clarification of figures, additional bioinformatic analyses, and clarification of bioinformatic analyses that had been submitted. EPA required sequence-based analyses of toxicity, allergenicity, and antinutrient potential of the PPV-CP and associated transgenes based on similarity to sequences known to exhibit these properties, and an individual volume addressing these issues was submitted. Under regulation (40 CFR 152.105) (http://cfr.vlex.com/vid/152-105-incomplete-applications-19815353), EPA is obliged to allow 75 days to address the deficiencies in the application. The level of analyses required to comply with the EPA request for additional information made it necessary that we request an extension of the Pesticide Registration Improvement Renewal Act (PRIA) due date (the date that EPA would complete the registration decision) which EPA granted. While the September 2007 request for additional information was being addressed, another request for additional information was received from EPA in February 2008. During this period meetings with EPA were held in order to clarify the requests and to discuss issues including the propagation, production and distribution of fruit trees, tree labeling and associated horticultural issues. Responses to the information requests of September 2007 and February 2008 along with hard copies of all cited references in the original submission and supplemental submissions were submitted to EPA in July 2008. On October 29, 2008 EPA published in the Federal Register (73 FR 64325) a Notice of Receipt announcing that IR-4 submitted on behalf of the USDA-ARS-AFRS (the applicant) an application to register a pesticide product containing a new active ingredient not included in any currently registered pesticide product (the PPCV-CP gene). Four comments were received during a 30 day comment period following the publication of the notice, all favorable. A petition (7E7231) seeking an exemption from the requirement of a tolerance for residues of the PPV-CP in stone fruit and almonds was filed by IR-4 on behalf of the UDSA-ARS-AFRS. EPA published a notice of filing of the petition in the Federal Register on November 14, 2008 (73 FR 67512) and the public was given a 30 day comment period. EPA received no comments on this notice. During the EPA review process we requested a number of conference calls and face-to-face meetings with EPA in order to obtain information on the status of the review and the status of the requested exemption of tolerance for the PPV-CP in stone fruits and almond. These meetings helped us to provide information to EPA that was relevant to their decision-making process. EPA informed us that an independent laboratory validation (ILV) of our proposed method for detecting the transgene in 'HoneySweet' leaves would be required and we began the process of soliciting a laboratory that the EPA considered appropriate. In

December 2009, EPA indicated a need to extend the PRIA date from January 8, 2010 to July 8, 2010. The need for this extension was the result of a new transparency requirement initiated by EPA which required a 30 day public comment period on the draft registration decision followed by a 60 day period during which the public would have the opportunity of submitting objections or hearing requests. The 'HoneySweet' petition although well underway and very close to a final decision, was not grandfathered-in but was subject to the process. Due to this new requirement and the need for EPA to review the draft ILV protocol, EPA proposed a 6 month PRIA extension. A 4 month (May 8, 2010) PRIA extension was negotiated. On April 1, 2010 the draft registration was published on the web (http://www.regulations.gov/#!docketDetail;D=EPA-HQ-OPP-2008-0742) with a comment period ending on April 30, 2010. Seventy eight comments were received; seventy six were highly supportive of registration, including some eloquently questioning the need for registration and the classification of 'HoneySweet plum as a biopesticide. Comments included opinions that the mechanism of resistance does not produce a PIP since no CP is produced and DNA has never been considered alone to be a pesticidal substance. The labeling of trees as pesticidal was also brought into question. It was suggested that mandatory labeling of 'HoneySweet' trees and propagative material as pesticidal (fruit would not be labeled) would cause substantial damage to the market for 'HoneySweet' and sets a precedent for future transgenic virus resistant crops to be treated "in the same unscientific and irrational manner." (for specific comments cited see http://www.regulations.gov/#!docketDetail;D=EPA-HQ-OPP-2008-0742). On May 7, 2010 EPA issued a 1 year conditional registration for 'HoneySweet' plum. A major condition of the registration to be fulfilled within 1 year was the ILV. At the time of conditional registration EPA agreed on the methodology in the protocol and the selection of the independent laboratory (Field Laboratory Services, Agricultural Marketing Service, Gastonia, NC) but the validation had not yet been performed. On November 2, 2010 the completed ILV was received by EPA and it was approved on January 13, 2011. The unconditional Sect. 12.3 registration was issued on August 8, 2011.

A final rule establishing the exemption from tolerance was effective on May 26, 2010 EPA-HQ-OPP-2008-0763; FRL-8826-9 (http://edocket.access.gpo.gov/2010/2010-12579.htm). This exemption clears the future use of PPV-CP genes for genetically engineered resistance to PPV in stone fruits and almonds whether the CP is expressed or not, without the necessity of seeking a tolerance level for PPV-CP.

12.5 Conclusions

At the time of this writing PPV continues to elude eradication efforts in Canada and is slowly spreading in New York State. Although federal and state authorities are working to prevent disease spread through culling and quarantine programs the multi-state detection of PPV clearly indicates that U.S. growers remain at risk from future PPV outbreaks. California produces 99 % of the U.S. plum supply and

40–60 % of the world supply of dried plums (prunes). The export value is $132 M. PPV presents a serious threat to this industry. The history of PPV spread world-wide demonstrates that conventional control methods such as chemical control of insects, quarantine, and even eradication efforts have proven to be costly and, in the long-term, unsuccessful. Disease-resistant fruit trees would provide the U.S. industry with a long-term, sustainable solution to the threat of PPV spread and would help to prevent the spread of PPV into susceptible native *Prunus* species which are virtually all susceptible (Damsteegt et al. 2006). There are few reports of naturally occurring high level, multi-strain resistance to PPV in most commercial *Prunus* species. Resistance has been reported in apricot (Ruiz et al. 2011) and hypersensitivity has been reported in plum (Hartmann and Petruschke 2002) and this mechanism can provide a reasonable level of resistance in plum if properly managed (Polák et al. 2005). We have demonstrated that genetic engineering can be an important source of high level and durable resistance against all known strains tested thus far. We have shown through a number of field studies the environmental safety of this technology (Capote et al. 2008; Fuchs et al. 2007; Zagrai et al. 2008, 2011). Nevertheless, the utilization of this demonstrated effective technology for the practical control of PPV has not occurred outside of the work with 'HoneySweet'. There are a number of reasons for this situation as discussed in the introduction to this chapter and elsewhere in this book. Clearly, the reticence of researchers to become involved in the regulatory arena is among these. Institutions supporting agricultural research need to find ways to encourage, support, and reward researchers who pursue regulatory approval efforts. The IR-4 Project (http://ir4.rutgers.edu/), represents a pathway for registration to public sector researchers and is currently assisting in the registration of other transgenic crops. Other organizations such as the Public-Sector Intellectual Property Resource for Agriculture (PIPRA) (http://www.pipra.org/) and Specialty Crop Regulatory Assistance (SCRA) (http://www.specialtycropassistance.org/) are also available to assist in navigating intellectual property and regulatory issues. When feasible, industry partners should be sought that have an interest in bringing a potential product through the regulatory process. Regulations should be science-based with clear submission criteria and should seek to minimize the cost and bureaucracy associated with submissions. The long-standing successes of virus control in squash and papaya (Oliver et al. 2011) and the current work with plum demonstrate the power and the safety of this approach. Institutional support, the commitment of researchers, clear, science-based regulatory frameworks that build upon a developing knowledge base, industry support, and public outreach are components that are now necessary to move this technology forward to improve agricultural production and its sustainability.

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References

Atanassov D (1932) Plum pox. A new virus disease. Ann Univ Sofia, Fac Agric Silv 11:49–69

Beachy RN, Loesh-Fries S, Tumer NE (1990) Coat protein-mediated resistance against virus infection. Annu Rev Phytopathol 28:451–474

Cambra M, Capote N, Myrta A, Llácer G (2006) Plum pox virus and the estimated costs associated with sharka disease. OEPP/EPPO Bull 36:202–204

Capote N, Pérez-Panadés J, Monzó C, Carbonell E, Urbaneja A, Scorza R, Ravelonandro M, Cambra M (2008) Assessment of the diversity and dynamics of Plum pox virus and aphid populations in transgenic European plums under Mediterranean conditions. Transgenic Res 17:367–377

Damsteegt VD, Scorza R, Stone AL, Schneider WL, Webb K, Demuth M, Gildow FE (2006) *Prunus* host range of *Plum pox virus* (PPV) in the United States by aphid and graft inoculation. Plant Dis 91:18–23

Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1990) Stable transformation of papaya via microprojective bombardment. Plant Cell Rep 9:189–194

Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1992) Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus. Biotechnology 10:1466–1472

Fuchs M, Cambra M, Capote N, Jelkmann W, Kundu J, Laval J, Martelli GP, Minafra A, Petrovic N, Pfeifffer P, Pompe-Novak M, Ravelonandro M, Saldarelli P, Stussi-Garaud C, Vigne E, Zagrai I (2007) Safety assessment of transgenic plums and grapevines expressing viral coat protein genes: new insights into real environmental impact of perennial plants engineered for virus resistance. J Plant Pathol 89:5–12

Gonsalves D (1998) Control of papaya ringspot virus in papaya – a case study. Annu Rev Phytopathol 36:415–437

Hartmann W, Petruschke M (2002) Sharka resistant plums and prunes by utilization of hypersensitivity. Acta Hortic 538:391–395

Hily J-M, Scorza R, Malinowski T, Zawadzka B, Ravelonandro M (2004) Stability of gene silencing-based resistance to *Plum pox virus* in transgenic plum (*Prunus domestica* L.) under field conditions. Transgenic Res 13:427–436

Hily J-M, Scorza R, Webb K, Ravelonandro M (2005) Accumulation of the long class of siRNA is associated eith resistance to *Plum pox virus* in a transgenic woody perennial plum tree. MPMI 18:794–799

Levy L, Damsteegt V, Welliver R (2000) First report of *Plum Pox Virus* (Sharka Disease) in *Prunus persica* in the United States. Plant Dis 8:202

Ling K, Namba S, Gonsalves C, Slightom JL, Gonsalves D (1991) Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein. Biotechnology 9:752–758

Malinowski T, Cambra M, Capote N, Zawadzka B, Gorris MT, Scorza R, Ravelonandro M (2006) Field trials of plum clones transformed with the *Plum pox virus* coat protein (PPV-CP) gene. Plant Dis 90:1012–1018

Mante S, Morgens PH, Scorza R, Cordts JM, Callahan AM (1991) *Agrobacterium*-mediated transformation of plum (*Prunus domestica* L.) hypocotyls slices and regeneration of transgenic plants. Biotechnology 9:853–857

Oliver JE, Tennant PF, Fuchs M (2011) Virus resistant transgenic horticultural crops: safety issues and lessons from risk assessment studies. In: Beiquan M, Scorza R (eds) Transgenic horticultural crops: challenges and opportunities EDS. Taylor and Francis, CRC Press, Boca Raton, pp 263–288

Polák J, Pívalová J, Svoboda J (2005) Prune cv. Jojo resistance to different strains of Plum pox virus. Plant Prot Sci 41:47–51

Ravelonandro M, Monsion M, Teycheney PY, Delbos R, Dunez J (1992) Construction of a chimeric viral gene expressing *plum pox virus* coat protein. Gene 120:167–173

Ravelonandro M, Scorza R, Bachelier JC, Labonne G, Levy L, Damsteegt V, Callahan AM, Dunez J (1997) Resistance of transgenic *Prunus domestica* to plum pox virus infection. Plant Dis 81:1231–1235
Ruiz EMV, Soriano JM, Romero C, Zhebentyayeva T, Terol J, Zuriaga E, Llácer G, Abbott AG, Badenes ML (2011) Narrowing down the apricot *Plum pox virus* resistance locus and comparative analysis with the peach genome syntenic region. Mol Plant Pathol 12:535–547
Scorza R, Levy L, Damsteegt V, Yepes LZ, Cordts J, Hadidi A, Slightom J, Gonsalves D (1995) Transformation of plum with the *Papaya ringspot virus* coat protein gene and reaction of transgenic plants to *Plum pox virus*. J Am Soc Hortic Sci 120:943–952
Scorza R, Callahan A, Levy L, Damsteegt V, Webb K, Ravelonandro M (2001) Post-transcriptional gene silencing in plum pox virus resistant transgenic European plum containing the plum pox potyvirus coat protein gene. Transgenic Res 10:201–209
Various authors (2006) Current status of *Plum pox virus* and sharka disease worldwide. OEPP/EPPO Bull 36:205–218
Zagrai I, Capote N, Ravelonandro M, Cambra M, Zagrai I, Scorza R (2008) Plum pox virus silencing of C5 transgenic plums is stable under challenge inoculation with heterologous viruses. J Plant Pathol 90:S1.63–S1.71
Zagrai I, Ravelonandro M, Gaboreanu I, Ferencz B, Scorza R, Zagrai L, Kelemen B, Pamfil D, Popescu O (2011) Transgenic plums expressing the Plum Pox Virus coat protein gene do not assist the development of PPV recombinants under field conditions. J Plant Pathol 93:159–165

Chapter 13
Genetically Engineered Insects – Regulatory Progress and Challenges

Luke S. Alphey and Camilla J. Beech

Abstract Genetically engineered insects (GE) represent a potentially valuable new tool in the control of insect pests both in agriculture and public health.

Insects that are currently being regulated for non-laboratory use are based on the development of the Sterile Insect Technique (SIT) for pest population suppression, using genetics to enhance or replace aspects of current SIT methods. Genetics-based improvements include the provision of a heritable marker, replacement of radiation-sterilization, large-scale sex separation and improved biosecurity. In the USA, open field trials of genetically engineered pink bollworm (*Pectinphora gossypiella*), a serious economic pest of cotton, have been taking place since 2006 and the first Environmental Impact Statement on any genetically engineered organism, under the National Environmental Protection Act (NEPA), was developed and approved for GE pink bollworm and fruit flies. This chapter will look at the regulatory process and data requirements for moving GE insects from laboratory to field testing, and the current status of regulations and guidance documents on GE insects in plant pest control programs. Additionally it will discuss areas for further development in regulation of GE insects. Regulatory risk – uncertainty in timescale, cost and outcome – is cited by developers, investors and potential users of GE insects as the single biggest concern and obstacle to the development and deployment of novel products in this area.

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Keywords Regulation • Genetically engineered insects • Environmental Impact Statement • Plant pests

13.1 Introduction to GE Insect Technology and Potential Agricultural Applications

Genetically-engineered (GE) insects are being developed for a wide range of purposes. This chapter focuses on agricultural applications involving field release of GE insects. Similar technology is being applied to mosquito vectors of human disease. Nonetheless one should recognise that the largest use of GE insects is laboratory-based study using *Drosophila melanogaster*, dwarfing all other uses of GE insects. A single major stock centre – the Bloomington Drosophila Stock Center at Indiana University – held 30,812 stocks at the end of 2010, expects to expand to 60–70,000 stocks and distributed almost 200,000 subcultures to other laboratories in 2010.[1] This is primarily for basic research, but in fact much of the genetic technology – and many of the scientists – involved in more applied work on agricultural pest insects and mosquitoes have their origins in *Drosophila*.

Drosophila melanogaster was the first insect for which genetic transformation methods were developed, meaning the ability to insert exogenous DNA into the insect's genome (Rubin and Spradling 1982; Spradling and Rubin 1982). Transformation of any other insect took another 13 years (Loukeris et al. 1995) due to surprising failure of the *Drosophila P* element vector system to work in other insects, and the further requirement to develop suitable markers. However, we now have transformation systems based on alternative transposons – principally *piggyBac*, but also *Minos, Hermes* and *mariner/mos1* – and fluorescent markers such as EGFP and DsRed which work across a very wide range of insects (reviewed by Handler and James 2000; Morrison et al. 2011). Genetic transformation is still a time-consuming, laborious and inefficient process, but can be considered 'routine' in the sense that for most insects simply generating the transgenic strains is no longer a major research endeavour.

Potential agricultural applications of GE insect technology can be categorised in several ways (Alphey 2009); one relates to phenotype. Pest insects can be attacked or beneficial insects protected. This might involve deleterious traits such as lethality or sterility in the case of pest insects, or advantageous traits such as chemical or pathogen resistance for beneficial insects. Considerable research has been directed at making mosquitoes less able to transmit human diseases such as malaria or dengue (Alphey et al. 2002) with some proof-of-principle successes (Corby-Harris et al. 2010; Franz et al. 2006; Ito et al. 2002; Kokoza et al. 2000); similar approaches may one day be applied to vectors of plant and animal diseases. More subtle changes will become possible in the future. For example, it has been suggested that malaria

[1] http://flystocks.bio.indiana.edu/Inst/history.htm. Accessed 7 Feb 2011.

transmission could be reduced by reducing the strong preference of key malaria vectors for biting humans relative to other potential blood sources (Takken and Costantini 2006). One could imagine similar modification for plant or animal disease pests, or indeed for pollinators and other beneficial organisms. However, such subtle adjustments are beyond the current state of the art. This is not so much because of a lack of ability to adjust gene expression, though the tools for this are still fairly crude, rather it is because we do not have a sufficiently detailed understanding of behavioural genetics to know what genetic changes to make to give a specific change in behaviour.

Another key dimension for categorising genetic engineering strategies relates to the degree to which the transgene system is expected or intended to persist or even spread in the environment. At one end of the spectrum lie sterile-insect strategies in which all progeny of the released insects die. The transgene therefore disappears from the environment within one pest generation. Use of such methods, analogous to the Sterile Insect Technique (SIT, Dyck et al. 2005; Knipling 1955), generally require periodic release to maintain a population of sterile insects in the program area. Nonetheless, at the end of the program, or if any adverse event is detected, simple cessation of releases will lead to very rapid elimination of the transgene from the environment by natural selection. Lethal/sterile traits are an extreme example; other traits in which some but not all individuals are affected might disappear somewhat less rapidly, but the principle is the same. It is also important to consider mutant or partially-deleted versions of the transgene – if any part can confer a selective advantage then there is a possibility that such a fragment might persist or spread, even though the intact transgene cannot. At the opposite end of the spectrum of persistence or invasiveness lie gene drive systems. These are genetic systems designed to increase in allele frequency and, generally, in geographic distribution. The design purpose is to spread through the target population a trait that is beneficial to humans but not of sufficient selective advantage to the recipient population to allow it to spread of its own accord. The most prominent example in the literature is inability to transmit a pathogen, for example malaria or dengue but also potentially plant and livestock pathogens. A gene drive system would then be coupled to such a refractoriness gene and 'drive' it through the target population despite the modest fitness penalty presumed to be associated with the refractoriness gene. One potential failure mode is immediately apparent – that the gene drive system may become uncoupled from its 'cargo' and spread without it (Curtis et al. 2006). Gene drive systems are artificial selfish DNA elements, often modelled on naturally-occurring ones. One paradigm is the *P* element, which spread through all major populations of *Drosophila melanogaster* around the world within a few decades of a presumed transfer from another *Drosophila* species (Houck et al. 1991; Silva and Kidwell 2000). This natural example illustrates both the power and the irreversibility of some types of gene drive systems.

Despite the ubiquity and spreading potential of transposons, it has proven surprisingly difficult to develop transposon-based gene drive systems. Several other designs have been proposed (Sinkins and Gould 2006), with proof-of-principle for at least one in *Drosophila* (Chen et al. 2007), but none are yet available even as working prototypes in pest insect species.

Though gene drive systems are seen as highly aggressive systems, – capable, like the *P* element – of transforming an entire species, this is not always the case. In the middle of the spectrum from minimal-persistence systems such as sterile-insect approaches to highly invasive gene drive systems lie systems that will persist in the environment for some time post-release but are not capable of global spread (Gould et al. 2008; Rasgon 2009). One interesting technology, using homing endonuclease genes, can be used to develop more or less aggressive genetic systems depending on the exact configuration (Burt 2003; Deredec et al. 2008).

13.2 Current Progress/Precedents in Moving GE Insects from Laboratory to Field

Despite the range of applications described above, significant progress beyond the laboratory has so far only been realised in respect of sterile-insect methods. In the classical Sterile Insect Technique (SIT), large numbers of insects of the target pest species are reared, sterilized and released into the target area. These compete for mates with the wild population; any wild female mating a sterile male has fewer viable offspring than she would otherwise have done and so the population in the next generation tends to be smaller than it would otherwise have been, though this can also be influenced by density-dependent effects (Barclay 2005; Yakob et al. 2008). If sufficient sterile insects can be released for a sufficient period then the target population will decline and collapse (Dyck et al. 2005; Knipling 1955). The SIT has been used successfully against a range of pest insects, notably the New World screwworm (*Cochliomyia hominivorax*) and several tephritid fruit flies such as the Mediterranean fruit fly (*Ceratitis capitata*). The SIT is a species-specific and environmentally-friendly method of pest control. A key attraction is that, unlike most non-genetic methods, it becomes progressively more powerful with decreasing population density of the target pest (Klassen 2009), making it an attractive component of integrated pest management. However its use is relatively limited due to a number of restrictions, some of which can potentially be overcome by use of genetics and genetic engineering.

Several significant improvements are potentially available through the use of genetic engineering (Alphey 2002, 2007; Handler 2002). These include (i) the provision of a heritable genetic marker – for example a fluorescent protein – to improve discrimination of sterile and wild insects in field monitoring (see below). (ii) 'Genetic sexing' – genetics based methods to facilitate or automate the separation of males and females on a large scale – is also highly desirable. For several species, including mosquitoes and tephritid fruit flies, biting or oviposition by the adult females is damaging, and sterile females are therefore likely to cause some harm. Furthermore, large-scale field studies have shown that male-only releases of radiation-sterilized Medflies are 3–5x more effective than the same number of males would be in a bisex release, i.e. if released without prior separation from sterile females

(Rendón et al. 2004). The sterile females may distract the sterile males from seeking and mating wild females. Methods for large-scale sex separation have therefore been sought for many years, indeed the experiment of Rendón et al. was only possible because of a genetic sexing strain made using classical genetics (Franz 2005). While such translocation-based strains can be very effective, they are extremely time-consuming to construct. Furthermore, since the special chromosomes required cannot be transferred between species, construction must start from scratch in each new species. Genetic engineering holds out the prospect of more widely applicable methods. Various systems have been developed or proposed for agricultural pests (Condon et al. 2007; Dafa'alla et al. 2010; Fu et al. 2007; Heinrich and Scott 2000; Thomas et al. 2000) and for mosquitoes (Alphey et al. 2010; Catteruccia et al. 2005; Fu et al. 2010; Papathanos et al. 2009). (iii) 'Genetic sterilization' – genetic methods to avoid the need for radiation-sterilization. Radiation impacts somatic cells as well as germline cells and gametes and is clearly damaging to the insects. The extent of this varies from one species to another, on the radiation dose, the developmental stage to which it is applied, and other factors (Andreasen and Curtis 2005; Bakri et al. 2005). In some instances a lower radiation dose may be required, either accepting partial fertility as a trade-off for improved performance (Helinski et al. 2006), or relying on inherited sterility in the next generation (Carpenter et al. 2005), a phenomenon particularly relevant to Lepidoptera. Radiation generates random dominant lethal mutations in the gametes of irradiated individuals, so that when these combine with a wild-type gamete the resulting zygote dies, typically early in development. This points to a potential genetic engineering alternative – using engineered heritable dominant lethal or sterile mutations in place of radiation (Alphey 2002; Catteruccia et al. 2009; Fryxell and Miller 1995). Such a system likely needs to be conditional to allow the strain to be reared despite the presence of the dominant lethal genetic system, though there may be ways around this (Windbichler et al. 2008). One such system is called RIDL® (Release of Insects carrying a Dominant Lethal genetic system (Thomas et al. 2000)); this is the first such system to have been tested in the field, as described below. Prototype strains have been developed for several dipteran and lepidopteran pests (Gong et al. 2005; Schetelig et al. 2009; Simmons et al. 2007). (iv) Biosecurity – the previous three areas each aim to improve effectiveness; there is additionally a potential improvement in terms of risk mitigation. In conventional SIT the wild type pest is present in large numbers in the mass-rearing facility, and is only converted to a biocontrol tool once it has been correctly and adequately sterilized. Though the SIT has a good record in this regard, batches of non-irradiated insects have occasionally been inadvertently released (e.g. del Valle 2003). Use of a repressible lethal system would substantially mitigate the risk of inadvertent release of mass-reared insects as the escapees, or their progeny, would die as a consequence of transgene expression (Alphey 2007).

Several prototype strains have advanced beyond the laboratory. One key question is whether laboratory strains will be able to compete effectively for mates in the wild. There are several reasons to think that they might not. The transgene itself might have deleterious effects – evidence on this is mixed (reviewed by Marrelli et al. 2006; Scolari et al. 2011); on the whole there seems little reason to think that

all transgenic strains will be compromised, but individual strains certainly might be. In addition, drift and selection may affect the strain background. Environmental factors such as rearing conditions and handling can also have a major impact on the performance of sterile males. Morrison et al. (2009) and Schetelig et al. (2009) found that transgenic strains of Medfly performed well in field-cage trials of male mating competitiveness, which is certainly encouraging. Lee et al. (2008, 2009) similarly found a RIDL strain of *Aedes aegypti* to be competitive in semi-field conditions. Wise de Valdez et al. (2011) went beyond mating competitiveness to show that another RIDL strain of *Aedes aegypti* could suppress – and indeed eliminate – target wild-type populations in large indoor cages.

Two transgenic strains have been used in open release trials, both with encouraging results These are a pink bollworm (*Pectinophora gossypiella*) engineered to express a fluorescent protein (Simmons et al. 2007, 2011) and a mosquito (*Aedes aegypti*) expressing a fluorescent protein and carrying a dominant repressible lethal genetic system (Harris et al. 2011).

13.3 Pink Bollworm

Radiation-based SIT is a component of an area-wide eradication program against the pink bollworm in the southwestern US (Antilla and Liesner 2008; Grefenstette et al. 2009; Tabashnik et al. 2010). As the program drives down moth populations in each area, and approaches local elimination, the need to accurately distinguish wild and transgenic moths becomes more critical. This is because a single putative wild moth in a trap can necessitate an expensive response; if that supposed wild moth was in fact a mis-identified sterile this is a waste of resources (Simmons et al. 2007). The sterile moths are currently marked using a food dye (Graham and Mangum 1971), but this is not considered to be 100% reliable (Hagler and Miller 2002; Simmons et al. 2011). Following the development of genetic transformation for pink bollworm (Peloquin et al. 2000), a transgenic strain expressing a red fluorescent marker (DsRed2) was developed to provide an improved marking system (Simmons et al. 2007). After laboratory development and testing, the strain was tested in outdoor field cages and in open releases. The strain used, OX1138B, has a heritable marker but not a genetic sterilization system, therefore the engineered moths were radiation-sterilized before field release, as per current SIT program operations and to allow fair comparisons with conventional sterile moths. Releases started in 2006; in 2008 about 15 million engineered moths were released from aircraft over 2,500 acres of cotton in Arizona. This series of experiments examined the survivorship (lifespan), dispersal and response to females (or a female sex pheromone) of the transgenic moths relative to non-transgenic controls, as well as the ability to suppress wild moth populations. The transgenic strain was found to perform well in respect of all these key performance parameters (Simmons et al. 2007) and also in its rearing properties. These experiments were conducted under permits from USDA APHIS Biotechnology Regulatory Services, including two Environmental Assessments (EA),

each of which led to a Finding of No Significant Impact (FONSI). The EAs were published in 2001 and 2006 for the confined studies and open release of genetically engineered pink bollworm (*Pectinophora gossypiella*). In both cases a Finding of No Significant Impact (FONSI) was issued and published in the Federal Register (66 FR 33226 and 71 FR 35408, respectively).

13.4 *Aedes aegypti*

Sterile-insect methods have great potential for mosquito control but conventional radiation-based methods have proven difficult to implement, due in part to the damaging effects of radiation on the mosquitoes (Alphey and Andreasen 2002; Alphey et al. 2010; Bellini et al. 2007; Benedict and Robinson 2003; Helinski et al. 2006, 2008). Genetic alternatives such as the RIDL system may therefore facilitate the use of sterile-male methods for mosquito control. An engineered RIDL strain of *Aedes aegypti*, the principal vector of dengue, was therefore developed and tested. This strain, OX513A, carries a repressible late-acting dominant lethal genetic system, and a fluorescent marker to aid identification of the transgenics (Phuc et al. 2007). Late-acting lethality is advantageous in species with significant density-dependent effects acting at a larval stage (Atkinson et al. 2007; Phuc et al. 2007); this is thought to be the case in *Aedes aegypti* (Dye 1984; but see also Legros et al. 2009). Following extensive laboratory, semi-field and computer analysis, open release experiments were initiated in the Cayman Islands (Harris et al. 2011).

In a first release period of 4 weeks (Nov–Dec 2009), just under 20,000 adult male *Aedes aegypti* were released to determine how they would interact with the environment and, in particular, the extent to which they would successfully mate wild females. The ability of sterile males to court and mate wild females is critical to sterile-male methods. The RIDL males were found to perform well relative to sterile males in previous, successful, SIT programs. Therefore, in 2010, a larger release trial was conducted at the same site to assess whether sustained release of RIDL males could suppress a wild *Aedes aegypti* population. Approximately 3.3 million RIDL males were released over 6 months, leading to a reduction in ovitrap index – a standard measure of *Aedes aegypti* populations – by 80% relative to control areas.[2] Since the release area was not isolated, at least some of the remaining population was likely due to immigration from adjacent untreated areas, especially of mated females (Alphey et al. 2010; Benedict and Robinson 2003; Dyck et al. 2005).

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[2] Data presented at 59th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Nov 2010; see also http://www.oxitec.com/wp-content/uploads/2010/11/Oxitec-MRCU-press-release.pdf. Accessed 21 Feb 2011.

13.5 Status of Current Regulations on GE Insects

It is useful perhaps to start by reminding ourselves of the purpose of having regulations for GE insects, which is to ensure the safety of the public and the protection of human health and the environment. Regulations are usually developed from national or international legislation and law and are implemented at a national or occasionally a regional level. Risk analysis, the weighing of risks against the potential benefits of their use, and the management of those risks are essential elements of the regulatory process.

Laboratory research with GE insects is routine, widespread and usually non-controversial, with well developed procedures and guidance in many countries (Benedict et al. 2003; Higgs 2004; Hirata and Filho 2002; Office of the Gene Technology Regulator (OGTR) 2006; Scott 2005). Risk assessment and management still however forms the cornerstone of laboratory processes with GE insects.

The regulatory requirements for the open release of GE insects has been widely debated for nearly two decades, starting in 1991 with the Vector Biology Network (WHO/TDR 1991), followed in 2002 and 2004 at the EU Frontis Workshops (Knols et al. 2004; Takken et al. 2002), at the International Atomic Energy Agency in 2006 (IAEA 2006) and again recently by the World Health Organization (WHO) (WHO 2009). The WHO concluded that there was no widely accepted regulatory or biosafety framework that provides guidance for all aspects of the implementation of genetically engineered insect technologies, but recognised that although national standards will take priority over other guidance, a framework that provided for standardisation of procedures and comparability of results could be useful for decision makers. The WHO document has been further developed in the last few years (2008–2011) regarding the regulation of GE insects (reviewed in Beech et al. 2009). Internationally, the Cartagena Protocol on Biosafety (CPB) has been widely adopted in many developing countries, although not in the USA, and requires signatories or Parties to take decisions regarding the import of Living Modified Organisms (LMOs) for intentional introduction into the environment. As such this forms part of the regulatory framework in many countries for the risk assessment and management of engineered organisms and will be used to assess genetically engineered insects for open release and trans-boundary movement. The CPB has recently focused on one specific type of GE insects; The Ad Hoc Technical Expert Group on Risk Assessment and Risk Management was convened after the 4th Conference of the Parties/Meeting Of the Parties (COP/MOP) meeting in 2009, and a sub-working group was formed to develop guidance documents on risk assessment and risk management of Living Modified Mosquitoes (LMM). After a series of online fora and working group meetings the sub-group report was finalised at 5th COP/MOP meeting in Nagoya, Japan in 2010. The Guidance is available online[3] (Fontes 2009). Some commentators have

[3] http://bch.cbd.int/onlineconferences/guidancedoc_ra_mosquitoes.shtml. Accessed 21 Feb 2011.

questioned whether this is an appropriate instrument for some GE insect releases (Angulo and Gilna 2008; Marshall 2010). The European Food Standards Authority has also recently published a report on "Defining Environmental Risk Assessment Criteria for Genetically Modified Insects to be placed on the EU market" (Benedict et al. 2010). This report describes developments in GE insects with particular reference to what might be released in the European Union in the next 10 year timeframe, and identifies potential adverse effects as well as methodologies that might be used to investigate these. The report concludes that the environmental risk assessment of GE insects should follow a case by case approach. It should be noted that in the EU the "environment" also considers the potential for adverse effects on human health.

In addition to these framework and guidance documents, several countries have made decisions under their own legislative frameworks regarding the open release of GE insects in the environment including the USA, Cayman Islands, Malaysia and Brazil.

In the USA, a review of the science and regulation of genetically modified insects was conducted in 2004 by the Pew Initiative on Food and Biotechnology (Pew Initiative on Food and Biotechnology 2004). The Pew report in particular gave an overview of the potential US laws and agencies that could cover the regulation of GE insects under the Coordinated Framework for the regulation of genetically engineered organisms, developed since 1986. These are summarised in Table 13.1. It also concluded that the USA federal government lacked a co-ordinated regulatory approach to ensure that all GE insects were reviewed for potential risks to human health, the environment, food safety and public health. Since 1986, as a matter of Federal Policy, the USA chose to regulate products of biotechnology as no different to similar products developed without the use of recombinant DNA methods as recombinant DNA is not a hazard *per se*. This was codified in the Co-ordinated Framework for Regulation of Biotechnology (US Office of Science and Technology Policy (OSTP) 1986) and validated by National Academy of Sciences in 1987 (NAS 1987). As a result biotechnology products in the USA are regulated on the basis of their characteristics and use rather than how they were made, and fall under the authority of existing regulations (Table 13.1).

This model has worked relatively well for GE crops, where hundreds of field trials have been reviewed and many products have now been deregulated (USDA 2012). However, it can be challenging to fit new categories of biotechnologies (no longer subject to regulation) into these existing laws, e.g. GE insects and specifically GE mosquitoes. However, there has been much progress both in the USA and internationally since the Pew report in 2004, with the USA making several regulatory decisions on GE insects under the Plant Protection Act (PPA) and the National Environmental Policy Act (NEPA). Recently the authors have learnt that the regulation of GE insects within the USA is under further review at the Office of Science and Technology Policy and may yet change again.

Table 13.1 Agency jurisdiction and law for GE insects in the USA

Agency	Law	Category	Transgenic insect use
FDA-CVM	Federal Food Drug and Cosmetic Act (FFDCA)	New animal drug	Genetically engineered animals (enforcement discretion for non-food animals falling under other oversight)
USDA-APHIS	Animal Health Protection Act (AHPA)	Animal pests	Animal disease vectors
USDA-APHIS	Plant Protection Act (PPA)	Plant pests	Plant pests
EPA	Federal Insecticide Fungicide and Rodenticide Act (FIFRA)	Pesticide	Pesticidal actions (including microbiological pest control agents but not macro-biologicals)

After Pew (2004)
FDA-CVM Food and Drug Administration, Center for Veterinary Medicine, *USDA-APHIS* United States Department of Agriculture, Animal and Plant Health Inspection Services, *EPA* Environmental Protection Agency

Internationally several countries have made their own regulatory decisions, approving open field releases of GE insects.[4]

13.6 Guidance and Decision Making for GE Insects in Plant Pest Control Programs

There appears to be clear oversight from USDA-APHIS, under the Plant Protection Act (PPA), where the use of the GE insect is to address a plant pest issue. The Plant Protection Act has broad authority to regulate plant pests, the definition of which includes insects and biological control agents (7 U.S.C §7701 et seq). It essentially prohibits the introduction or movement of any potential plant pest unless USDA has granted a permit. In direct contrast to GE crops, which are eventually deregulated (no longer subject to regulation) provided that they have met a range of requirements and performance standards, GE insects that are plant pests may continue to be regulated regardless of the scale at which they are released, as they will likely still be considered potential plant pests. This has yet to be determined, as no application has been submitted with a request for deregulated status at the time of writing.

[4] Cayman Islands risk assessment for genetically modified mosquitoes: http://www.parliament.uk/deposits/depositedpapers/2011/DEP2011-0053.pdf Malaysian government opinion on open release of GM mosquitoes: http://bch.cbd.int/database/record-v4.shtml?documentid=101480 Brazilian government opinion on open release of GM mosquitoes (in Portuguese): http://www.jusbrasil.com.br/diarios/23935599/dou-secao-1-17-12-2010-pg-48

The USA has already chosen to regulate GE pink bollworm in open field tests under the authority of the PPA. Significant precedent and experience has been built up, in both the regulated community and within USDA. This precedent and experience leads to a level of predictability – in terms of informational requirements, timescales and outcomes – which is crucial to the regulated community. Regulatory risk – uncertainty in timescale, cost and outcome – is cited by developers, investors and potential users of GE insects as the single biggest concern and obstacle to the development and deployment of novel products in this area.

Where the GE insect is a plant pest there is a wide range of Guidance documents already in existence that may be applicable or could be adopted for national regulation. The International Plant Protection Convention (IPPC) International Standards for Phytosanitary Measures (ISPM) have several guidances that could be adapted:

ISPM02: (2007) Framework for Pest risk analysis[5]
ISPM03: (2005) Guidelines for the shipment, import and release of biological control agents and other beneficial organisms[6]
ISPM11: (2004) Pest risk analysis for quarantine pests including analysis of environmental risks and living modified organisms[7].

Many of the features of these international guidance documents were incorporated in a regional standard adopted by the North American Plant Protection Organization (NAPPO, comprising the USA, Canada and Mexico), RSPM 27 published in 2007.

Confined field release definition from NAPPO RSPM 27

> Release of organisms into the environment under specific conditions and restrictions intended to prevent establishment in, or control spread into the environment, and/or limit the unintended interactions with the environment, of the organisms and any progeny derived from them.

This is further elaborated in the RSPM 27 text:

> Appropriate confinement conditions may consist of any one or a combination of the following confinement measures:
>
> - physical,
> - biological,
> - temporal, and/or
> - geographic

[5] https://www.ippc.int/index.php?id=1110798&tx_publication_pi1[showUid]=184204&frompage=13399&type=publication&subtype=&L=0#item. Accessed 21 Feb 2011.

[6] https://www.ippc.int/index.php?id=13399&tx_publication_pi1[showUid]=76047. Accessed 21 Feb 2011.

[7] https://www.ippc.int/index.php?id=13399&tx_publication_pi1[showUid]=34163. Accessed 21 Feb 2011.

NAPPO RSPM 27 was developed to provide guidance on the importation and confined field release of transgenic arthropods that are known plant pests or have the potential to affect plant health. This was one of the first Guidance documents produced that was specific to GE insects. It also includes transgenic arthropods for biological control. This regional standard is or needs to be implemented in national legislation in the three member countries (USA, Canada and Mexico). It is worth noting that the definition of confined release (see text box) in this standard is broad and could include field release of transgenic arthropods that were biologically confined as in the case of genetically sterile male arthropods for Sterile Insect Technique applications.

13.7 Development of an Environmental Impact Statement

In addition to NAPPO RSPM 27, another key document produced by USDA-APHIS regulators, is the Environmental Impact Statement (EIS)[8] on "Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programs". This was developed to analyze the potential use of engineered strains with heritable markers and/or autocidal genetic systems (e.g. RIDL) in pest control programs. Four species were considered – three tephritid fruit flies (Mediterranean fruit fly (*Ceratitis capitata*), Mexican fruit fly (*Anastrepha ludens*), and Oriental fruit fly (*Bactrocera dorsalis*)) and one moth, pink bollworm (*Pectinophora gossypiella*). However, the considerations of molecular technology and genetic principles are likely to be widely applicable, at least to this class of genetic modification. The Final EIS (FEIS) was published in October 2008 and Record of Decision in May 2009, making it the first EIS to be completed for any genetically engineered organism. The FEIS examined the potential environmental consequences of the incorporation of genetically engineered fruit fly or pink bollworm into existing area-wide Sterile Insect Technique (SIT) pest control programs for these insect species. This was compared with alternatives of either continuing as at present ('no action') or expanding the existing program without incorporating genetically engineered strains ('expansion of existing programs'). The FEIS was prepared under the National Environmental Policy Act (NEPA) and included extensive consultation comprising three public comment periods and five public meetings. The comments received during these consultations are included in an Appendix to the FEIS. Development of the EIS has further described by Robert Rose, one of the contributors (Rose 2009). The Record of Decision[9] states that

> *After a thorough evaluation of the potential impacts of the alternatives considered in the FEIS, APHIS has decided to integrate the use of genetically engineered insects into the sterile insect technique used in agency plant pest control programs.*

[8] http://www.aphis.usda.gov/plant_health/ea/geneng.shtml

[9] Federal Register Vol. 74 No 87, p21314-6, May 7, 2009, also available at http://www.aphis.usda.gov/plant_health/ea/geneng.shtml

This was based on a finding that this incorporation of genetically engineered insects is the environmentally preferable alternative:

> *The environmentally preferable alternative for the use of sterile insect technique in plant pest control programs is the alternative that minimizes potential impacts to human health, non-target species, and environmental quality. Among the alternatives considered in this EIS, the preferred alternative, which involves integration of genetically engineered insects into programs, is also the environmentally preferable alternative…*
>
> *…The potential environmental impacts from methods under alternatives other than the preferred alternative are reduced under the preferred alternative to the extent that genetically engineered insects are incorporated. For example, the use of genetically engineered insects has the potential to decrease the need for insecticide applications, to decrease the need to produce both male and female insects for use in sterile insect releases, to increase production of males that are more competitive in mating than radiation-sterilized males, and to eliminate the need to use, operate, and maintain strong gamma radiation sources.*
>
> *…integration of genetically engineered insects into programs, provides program managers with several methods for pest risk reduction in an environmentally safe and efficient manner.*

This FEIS is likely to be relevant and influential for all GE insect releases over the next several years. The principal limitations relate to scope. Though only four species were selected, these are from two different Orders. Other species may have particular issues not covered by the FEIS, such as human biting propensity in the case of mosquitoes. This is unlikely to make the FEIS irrelevant, but some additional analysis may be required. The FEIS may be less useful for genetic systems with properties very different to those it explicitly covers. The FEIS covers only heritable markers and autocidal genetic systems; in particular it does not cover invasive or self-sustaining genetic systems such as gene drive systems. Such systems are quite different in their behavior and the FEIS will be less useful as a guide in this area. Given that, at time of writing more than 2 years after completion of the FEIS, there are still no working prototypes of such systems in any major pest insect, to attempt to include them would probably have been premature. Indeed such an outcome was never the purpose of this document.

13.8 Areas for Further Development

As noted above, invasive or self-sustaining genetic systems represent a challenge for regulators in the future. An interesting analogy may be drawn with classical biological control, in which an exotic predator or parasitoid is released with the hope that it will establish and thereby suppress the target pest. Once established, exotic biological control agents are typically impossible to eliminate and may spread beyond their initial release area – indeed this is often the intention. In each of these respects such classical biological control somewhat resembles a self-sustaining genetic system.

An even closer analogy can be drawn with a new genetic strategy being developed for mosquitoes. This is the use of an intracellular bacterium, *Wolbachia pipientis*. Versions of this bacterium are found infecting many arthropod species, but their

properties are strain- and host-specific. Strains from *Drosophila* have been shown to make *Aedes aegypti* and other mosquitoes refractory (resistant) to infection by a range of pathogens (Hedges et al. 2008; Kambris et al. 2009; Moreira et al. 2009). *Wolbachia* itself does not spread by horizontal transfer – infection – from one individual to another, rather it is a reproductive parasite that distorts its host's reproductive biology and inheritance patterns to allow itself to spread through a population by exclusively vertical transmission from mother to offspring. In essence it is a selfish DNA element (Burt and Trivers 2006). The artificial infection of *Aedes aegypti* with an alien strain of *Wolbachia*, and the use of the selfish-DNA characteristics of this to drive a desirable trait (resistance to dengue) through a wild mosquito population is therefore indistinguishable in broad effect from the use of an invasive gene drive system. One key regulatory difference, however, is that this artificial infection can be achieved without the use of recombinant DNA technology, which means this approach is in something of a regulatory vacuum. It is striking, and perhaps a little incongruous, that adding part of the genome of this bacterium to the insect would lead to much stronger regulatory oversight than does adding the whole of the bacterial genome. For GE insects, a key concern and focus of risk assessment has been the likelihood and consequence of the novel genetic element spreading and establishing beyond the immediate release area. However, a risk assessment that was performed prior to a release of *Wolbachia*-infected *Aedes aegypti* in northern Australia (Murphy et al. 2010) did not include explicit discussion of the possibility of spread of the *Wolbachia* parasite to *Aedes aegypti* populations outside the release area. This highlights the very different regulatory approaches taken to these technologies, despite their closely related phenotypic consequences.

13.8.1 Translation of Regulatory Approvals to Implementation

Regulators and policymakers should understand the impact of their regulations in terms of a risk/benefit equation. This is particularly important when considering genetically engineered disease vectors where a potential benefit for human health may be realised. Regulators should consider performance based regulations that allow applicants to address safety requirements in innovative and flexible ways, while respecting the goals of the regulatory process. Provision should also be made to allow the risk assessment and regulatory process to be varied on the receipt of new information that could impact the risk assessment. It may be a case that certain activities become so routine and the risks so well known that provision should also be given to reduce the regulatory requirements for certain activities. Predictability and transparency in regulation – in terms of informational requirements, timescales and outcomes, is crucial to implementation of GE insects in the future and will build confidence in decision making, both within the regulated community and the general public, allowing novel insect control solutions to address the increasing threats of insect borne diseases in both public health and agriculture.

References

Alphey L (2002) Re-engineering the sterile insect technique. Insect Biochem Mol Biol 32: 1243–1247

Alphey L (2007) Engineering insects for the sterile insect technique. In: Vreysen M, Robinson A, Hendrichs J (eds) Area-wide control of insect pests: from research to field implementation. Springer, Dordrecht, pp 51–60

Alphey L (2009) Natural and engineered mosquito immunity. J Biol 8:40

Alphey L, Andreasen MH (2002) Dominant lethality and insect population control. Mol Biochem Parasitol 121:173–178

Alphey L, Beard B, Billingsley P, Coetzee M, Crisanti A, Curtis CF, Eggleston P, Godfray C, Hemingway J, Jacobs-Lorena M et al (2002) Malaria control with genetically modified vectors. Science 298:119–121

Alphey L, Benedict MQ, Bellini R, Clark GG, Dame D, Service M, Dobson S (2010) Sterile-insect methods for control of mosquito-borne diseases – an analysis. Vector Borne Zoonotic Dis 10:295–311

Andreasen MH, Curtis CF (2005) Optimal life stage for radiation sterilization of Anopheles for sterile insect releases. Med Vet Entomol 19:238–244

Angulo E, Gilna B (2008) When biotech crosses borders. Nat Biotechnol 26:277–282

Antilla L, Liesner L (2008) Program advances in the eradication of pink bollworm, *Pectinophora gossypiella*, in Arizona cotton. Paper presented at 2008 Beltwide cotton conferences. National Cotton Council of America, Nashville

Atkinson MP, Su Z, Alphey N, Alphey LS, Coleman PG, Wein LM (2007) Analyzing the control of mosquito-borne diseases by a dominant lethal genetic system. Proc Natl Acad Sci USA 104:9540–9545

Bakri A, Mehta K, Lance DR (2005) Sterilizing insects with ionizing radiation. In: Dyck VA, Hendrichs J, Robinson AS (eds) Sterile insect technique: Principles and practice in area-wide integrated pest management. Springer, Dordrecht, pp 233–268, Available at http://www.springerlink.com/content/978–1–4020–4050–4/front-matter.pdf

Barclay HJ (2005) Mathematical models for the use of sterile insects. In: Dyck VA, Hendrichs J, Robinson AS (eds) Sterile insect technique: Principles and practice in area-wide integrated pest management. Springer, Dordrecht, pp 147–174, ISBN-10 1-4020-4050-4 (HB)

Beech C, Vasan S, Quinlan M, Capurro ML, Alphey L, Bayard V, Bouare M, Mcleod-Corena M, Kittaypong P, Lavery J et al (2009) Deployment of innovative genetic vector control strategies: progress on regulatory and biosafety aspects, capacity building and development of best practice guidance. Asia Pac J Mol Biol Biotechnol 17:75–85

Bellini R, Calvitti M, Medici A, Carrieri M, Celli G, Maini S (2007) Use of the sterile insect technique against Aedes albopictus in Italy: first results of a pilot trial. In: Vreysen MB, Robinson AS, Hendrichs J (eds) Area-wide control of insect pests. Springer, Dordrecht, pp 505–515

Benedict M, Eckerstorfer M, Franz G, Gaugitsch H, Greiter A, Heissenberger A, Knols B, Kumschick S, Nentwig W, Rabitsch W (2010) Defining environmental risk assessment criteria for genetically modified insects to be placed on the EU market. Environment Agency Austria (Umweltbundesamt), Available at http://www.efsa.europa.eu/en/supporting/doc/71e.pdf

Benedict MQ, Robinson AS (2003) The first releases of transgenic mosquitoes: an argument for the sterile insect technique. Trends Parasitol 19:349–355

Benedict MQ, Tabachnick WJ, Higgs S, Azad AF, Beard CB, Beier JC, Handler AM, James AA, Lord CC, Nasci RS, Olson KE et al (2003) Arthropod containment guidelines. Version 3.1. Vector Borne Zoonotic Dis 3(2):57–98

Burt A (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proc Biol Sci 270:921–928

Burt A, Trivers R (2006) Genes in conflict: the biology of selfish genetic elements. Belknap Press/ Harvard University Press, Cambridge

Carpenter JE, Bloem S, Marec F (2005) Inherited sterility in insects. In: Dyck VA, Hendrichs J, Robinson AS (eds) Sterile insect technique: Principles and practice in area-wide integrated pest management. Springer, Dordrecht, pp 115–146, ISBN-13 978-1-4020-4050-4 (HB)

Catteruccia F, Benton J, Crisanti A (2005) An *Anopheles* transgenic sexing strain for vector control. Nat Biotechnol 23:1414–1417

Catteruccia F, Crisanti A, Wimmer E (2009) Transgenic technologies to induce sterility. Malar J 8:S7

Chen C-H, Huang H, Ward CM, Su JT, Schaeffer LV, Guo M, Hay BA (2007) A synthetic maternal-effect selfish genetic element drives population replacement in Drosophila. Science 316:597–600

Condon K, Condon G, Dafa'alla T, Fu G, Phillips C, Jin L, Gong P, Alphey L (2007) Genetic sexing through the use of Y-linked transgenes. Insect Biochem Mol Biol 37:1168–1176

Corby-Harris V, Drexler A, Watkins de Jong L, Antonova Y, Pakpour N, Ziegler R, Ramberg F, Lewis EE, Brown JM, Luckhart S et al (2010) Activation of *Akt* signaling reduces the prevalence and intensity of malaria parasite infection and lifespan in *Anopheles stephensi* Mosquitoes. PLoS Pathog 6:e1001003

Curtis C, Coleman PG, Kelly DW, Campbell-Lendrum DH (2006) Advantages and limitations of transgenic vector control: sterile males versus gene drivers. In: Boëte C (ed) Genetically modified mosquitoes for malaria control. Landes Bioscience, Austin

Dafa'alla T, Fu G, Alphey L (2010) Use of a regulatory mechanism of sex determination in pest insect control. J Genet 80:301–305

del Valle J (2003) New World screwworm (*Cochliomyia hominivorax*) in Mexico. Dis Inform 16:49–50

Deredec A, Burt A, Godfray H (2008) Population genetics of using homing endonuclease genes in vector and pest management. Genetics 179:2013–2026

Dyck VA, Hendrichs J, Robinson AS (eds) (2005) Sterile insect technique: Principles and practice in area-wide integrated pest management. Springer, Dordrecht, ISBN-10 1-4020-4051-2 (e-book)

Dye C (1984) Models for the population dynamics of the yellow fever mosquito, *Aedes aegypti*. J Anim Ecol 53:247–268

Fontes E (2009) Risk assessment and risk management under the Cartagena protocol on biosafety. Asia Pac J Mol Biol Biotechnol 17:97–98

Franz G (2005) Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique. In: Dyck VA, Hendrichs J, Robinson AS (eds) Sterile insect technique: Principles and practice in area-wide integrated pest management. Published by Springer, Dordrecht, pp 427–451

Franz A, Sanchez-Vargas I, Adelman Z, Blair C, Beaty B, James A, Olson K (2006) Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. Proc Natl Acad Sci USA 103:4198–4203

Fryxell K, Miller T (1995) Autocidal biological control: a general strategy for insect control based on genetic transformation with a highly conserved gene. J Econ Entomol 88:1221–1232

Fu G, Condon KC, Epton MJ, Gong P, Jin L, Condon GC, Morrison NI, Dafa'alla TH, Alphey L (2007) Female-specific insect lethality engineered using alternative splicing. Nat Biotechnol 25:353–357

Fu G, Lees RS, Nimmo D, Aw D, Jin L, Gray P, Berendonk TU, White-Cooper H, Scaife S, Kim Phuc H et al (2010) Female-specific flightless phenotype for mosquito control. Proc Natl Acad Sci USA 107:4550–4554

Gong P, Epton M, Fu G, Scaife S, Hiscox A, Condon K, Condon G, Morrison N, Kelly D, Dafa'alla T et al (2005) A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. Nat Biotechnol 23:453–456

Gould F, Huang Y, Legros M, Lloyd A (2008) A killer-rescue system for self-limiting gene drive of anti-pathogen constructs. Proc R Soc B 275:2823–2829

Graham HM, Mangum GL (1971) Larval diets containing dyes for tagging pink bollworm. J Econ Entomol 64:377–379

Grefenstette B, El-Lissy O, Staten RT (2009) Pink bollworm eradication plan in the US (USDAAPHIS), Available at http://www.aphis.usda.gov/plant_health/plant_pest_info/cotton_pests/downloads/pbw-erad-plan2-09.pdf
Hagler JR, Miller E (2002) An alternative to conventional insect marking techniques: detection of a protein mark of pink bollworm by ELISA. Entomol Exp Appl 103:1–9
Handler A (2002) Prospects for using genetic transformation for improved SIT and new biocontrol methods. Genetica 116:137–149
Handler A, James A (2000) Insect transgenesis: methods and applications. CRC Press, Boca Raton
Harris AF, Nimmo D, McKemey AR, Kelly N, Scaife S, Beech C, Petrie W, Alphey L (2011) Field performance of engineered male mosquitoes. Nat Biotechnol 29(11):1034–1037
Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) *Wolbachia* and virus protection in insects. Science 322:702
Heinrich J, Scott M (2000) A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. Proc Natl Acad Sci USA 97:8229–8232
Helinski M, Parker A, Knols BGJ (2006) Radiation-induced sterility for pupal and adult stages of the malaria mosquito *Anopheles arabiensis*. Malar J 5:41
Helinski MEH, Hassan MM, El-Motasim WM, Malcolm CA, Knols BGJ, El-Sayed B (2008) Towards a sterile insect technique field release of *Anopheles arabiensis* mosquitoes in Sudan: Irradiation, transportation, and field cage experimentation. Malar J 7:65
Higgs S (2004) The containment of arthropod vectors. University Press of Colorado, Niwot
Hirata MH, Filho JM (2002) Manual de Biossegurança. Manole, São Paulo, p 466
Houck M, Clark J, Peterson K, Kidwell M (1991) Possible horizontal transfer of Drosophila genes by the mite Proctolaelaps regalis. Science 253:1125–1128
IAEA (2006) Status and risk assessment of the use of transgenic arthropods in plant protection. Paper presented at: proceedings of a technical meeting organised by the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture and the Secretariat of the International Plant Protection Convention, Rome
Ito J, Ghosh A, Moreira LA, Wimmer EA, Jacobs-Lorena M (2002) Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. Nature 417:452–455
Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. Science 326:134–136
Klassen W (2009) Introduction: development of the sterile insect technique for African malaria vectors. Malar J 8:I1
Knipling EF (1955) Possibilities of insect control or eradication through the use of sexually sterile males. J Econ Entomol 48:459–469
Knols BG, Louis C, Bogers RJ (2004) Bridging laboratory and field research for genetic control of disease vectors. Paper presented at Joint WHO/TDR, NIAID, IAEA and Frontis workshop on bridging laboratory and field research for genetic control of disease vectors, Nairobi
Kokoza V, Ahmed A, Cho W-L, Jasinskiene N, James A, Raikhel A (2000) Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. Proc Natl Acad Sci USA 97:9144–9149
Lee H, Vasan S, Nazni W, Shanaz M (2008) Scientific report on the innovative application of *Aedes aegypti* RIDL-sterile insect technique to combat dengue and chikungunya in Malaysia. Institute of Medical Research, Kuala Lumpur
Lee HL, Joko H, Nazni WA, Vasan SS (2009) Comparative life parameters of transgenic and wild strain of *Aedes aegypti* in the laboratory. Dengue Bull 33:103–114
Legros M, Lloyd AL, Huang Y, Gould F (2009) Density-dependent intraspecific competition in the larval stage of Aedes aegypti (Diptera: Culicidae): revisiting the current paradigm. J Med Entomol 46:409–419
Loukeris TG, Livadaras I, Arca B, Zabalou S, Savakis C (1995) Gene transfer into the medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. Science 270:2002–2005

Marrelli MT, Moreira CK, Kelly D, Alphey L, Jacobs-Lorena M (2006) Mosquito transgenesis: what is the fitness cost? Trends Parasitol 22:197–202

Marshall JM (2010) The Cartagena protocol and genetically modified mosquitoes. Nat Biotechnol 28:896–897

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M et al (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. Cell 139:1268–1278

Morrison NI, Segura DF, Stainton KC, Fu G, Donnelly CA, Alphey LS (2009) Sexual competitiveness of a transgenic sexing strain of the Mediterranean fruit fly, *Ceratitis capitata*. Entomol Exp Appl 133:146–153

Morrison NI, Franz G, Koukidou M, Miller TA, Saccone G, Alphey LS, Beech CJ, Nagaraju J, Simmons GS, Polito LC (2011) Genetic improvements to the sterile insect technique for agricultural pests. Asia Pac J Mol Biol Biotechnol 18:275–295

Murphy B, Jansen C, Murray J, De Barro P (2010) Risk analysis on the Australian release of Aedes aegypti (L) containing Wolbachia. CSIRO, Canberra, Australia, p 103, P.O. Box 17, 3300 AA Dordrecht, The Netherlands

NAS (1987) Introduction of recombinant DNA-engineered organisms into the environment: key issues. National Academies Press, Washington, DC

Office of the Gene Technology Regulator (OGTR) (2006) Guidelines for the certification of a physical containment level 2 arthropod facility. Version 2.1, Available at <http://www.health.gov.au/internet/ogtr/publishing.nsf/Content/PC2-7/$FILE/PC2ARTHv2-1.rtf>

Papathanos P, Bossin H, Benedict M, Catteruccia F, Malcolm C, Alphey L, Crisanti A (2009) Sex separation strategies: past experience and new approaches. Malar J 8:S5

Peloquin JJ, Thibault ST, Staten R, Miller TA (2000) Germ-line transformation of pink bollworm (Lepidoptera: Gelechiidae) mediated by the *piggyBac* transposable element. Insect Mol Biol 9:323–333

Pew Initiative on Food and Biotechnology (2004) Bugs in the system? Pew Initiative on Food and Biotechnology, Washington, DC, USA, Available at www.springeronline.com

Phuc HK, Andreasen MH, Burton RS, Vass C, Epton MJ, Pape G, Fu G, Condon KC, Scaife S, Donnelly CA et al (2007) Late-acting dominant lethal genetic systems and mosquito control. BMC Biol 5:11

Rasgon JL (2009) Multi-Locus Assortment (MLA) for transgene dispersal and elimination in mosquito populations. PLoS One 4:e5833

Rendón P, McInnis D, Lance D, Stewart J (2004) Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. J Econ Entomol 97:1547–1553

Rose R (2009) A short note on the final environmental impact statement–October 2008: use of genetically engineered fruit fly and pink bollworm in APHIS plant pest control programs. Asia Pac J Mol Biol Biotechnol 17:85–89

Rubin G, Spradling A (1982) Genetic transformation of Drosophila with transposable element vectors. Science 218:3448–3453

Schetelig M, Caceres C, Zacharopoulou A, Franz G, Wimmer EA (2009) Conditional embryonic lethality to improve the sterile insect technique in *Ceratitis capitata* (Diptera: Tephritidae). BMC Biol 7:4

Scolari F, Siciliano P, Gabrieli P, Gomulski L, Bonomi A, Gasperi G, Malacrida A (2011) Safe and fit genetically modified insects for pest control: from lab to field applications. Genetica 139:41–52

Scott TW (2005) Containment of arthropod disease vectors. Inst Lab Anim Res J 46(1):53–61

Silva J, Kidwell M (2000) Horizontal transfer and selection in the evolution of P elements. Mol Biol Evol 17:1542–1557

Simmons G, Alphey L, Vasquez T, Morrison N, Epton M, Miller E, Miller T, Staten R (2007) Potential use of a conditional lethal transgenic pink bollworm *Pectinophora gossypiella* in area-wide eradication or suppression programmes. In: Vreysen MB, Robinson AS, Hendrichs J (eds) Area-wide control of insect pests. Springer, Dordrecht, pp 119–123

Simmons GS, McKemey AR, Morrison NI, O'Connell S, Tabashnik BE, Claus J, Fu G, Tang G, Sledge M, Walker AS et al (2011) Field performance of a genetically engineered strain of pink bollworm. PLoS One 6(9):e24110

Sinkins S, Gould F (2006) Gene drive systems for insect disease vectors. Nat Rev Genet 7:427–435

Spradling A, Rubin G (1982) Transposition of cloned P elements into Drosophila germ line chromosomes. Science 218:341–347

Tabashnik BE, Sisterson MS, Ellsworth PC, Dennehy TJ, Antilla L, Liesner L, Whitlow M, Staten RT, Fabrick JA, Unnithan GC et al (2010) Suppressing resistance to Bt cotton with sterile insect releases. Nat Biotechnol 28:1304–1307

Takken W, Costantini C (2006) The genetics of vector-host interactions: alternative strategies for genetic engineering for malaria control. In: Boëte C (ed) Genetically modified mosquitoes for malaria control. Landes Bioscience, Austin

Takken W, Scott TW, Bogers RJ (2002) Ecological aspects for application of genetically modified mosquitoes. Paper presented at Frontis workshop on ecological challenges concerning the use of genetically modified mosquitoes for disease control, Wageningen

Thomas DD, Donnelly CA, Wood RJ, Alphey LS (2000) Insect population control using a dominant, repressible, lethal genetic system. Science 287:2474–2476

US Office of Science and Technology Policy (OSTP) (1986) Coordinated framework for regulation of biotechnology: announcement of policy and notice for public comment. Fed Regist:203–223, 393

USDA (2012) Determinations of non-regulated status (online) Available at http://www.aphis.usda.gov/biotechnology/not_reg.html. Accessed 28 June 2012

WHO (2009) Planning meetings on progress and prospects for the use of genetically modified mosquitoes to prevent disease transmission: meeting 1 technical consultations on the current status and planning for future development, Geneva

WHO/TDR (1991) Report of the meeting "Prospects for malaria control by genetic manipulation of its vectors". Paper presented at Vector biology meeting, Tuscon, Arizona, TDR/BCV/MAL-ENT/91

Windbichler N, Papathanos PA, Crisanti A (2008) Targeting the X chromosome during spermatogenesis induces Y chromosome transmission ratio distortion and early dominant embryo lethality in *Anopheles gambiae*. PLoS Genet 4:e1000291

Wise de Valdez MR, Nimmo D, Betz J, Gong H-F, James AA, Alphey L, Black WC IV (2011) Genetic elimination of dengue vector mosquitoes. In: Proceedings of the National Academy of Sciences (USA) published ahead of print 7 Mar 2011

Yakob L, Alphey L, Bonsall M (2008) *Aedes aegypti* control: the concomitant role of competition, space and transgenic technologies. J Appl Ecol 45:1258–1265

Chapter 14
Regulation of Genetically Engineered Animals

Elizabeth A. Maga and James D. Murray

Abstract The advent of genetic engineering (GE) techniques to produce transgenic animals in the early 1980s held the promise of being able to make precise changes to the genome of an animal to improve production traits and health in a faster and more specific fashion than possible with traditional breeding and selection. However, almost 30 years later, there are still no GE animal derived food products approved for use world-wide. The first, and to date only, product from a GE animal to be approved for use was human antithrobmin (A-Tryn®) produced in the milk of transgenic dairy goats as a human pharmaceutical. This is in stark contrast to the applications of GE plants, which are numerous and were approved very early in their development compared to GE animals. Why is this so? This chapter will give some perspective on the regulatory process for GE animals with respect to research and development of biotechnology-derived products and address issues that have been holding back the implementation of GE livestock. Topics will include methods for producing GE livestock, agricultural and medical applications of GE animals, regulatory guidelines in the US and factors influencing the development and implementation of GE animals.

Keywords Animals • Genetic engineering • Lentivirus • *Piggyback* • Transfection • Transposon

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14.1 Methods for Making Genetically Engineered Animals

Today, transgenic animals can be generated via a variety of methods including pronuclear microinjection, transfection of embryonic stem (ES) cells, somatic cell nuclear transfer (SCNT) using GE cells, transposons and retroviral vectors that allow for many types of gene modifications. Transgenic animals were first generated via the technique of pronuclear microinjection whereby transgene DNA was injected into one pronucleus of one-cell mouse zygotes that were then surgically transferred into synchronous recipient females (Gordon and Ruddle 1981). Resulting offspring were then screened for the presence and expression of the transgene and bred to non-transgenic animals to establish hemizygous lines for study. Pronuclear microinjection can be applied to a wide range of mammalian species but results in the random integration of the transgene into the chromosome of the zygote. Therefore, only gene addition is allowed and the promoter and all other regulatory elements required to achieve the desired expression must be included in the transgene construct.

The use of ES cells allowed for more precise gene modifications to be made prior to the generation of an animal. ES cells are undifferentiated pluripotent cells derived from the inner cell mass (ICM) of early embryos (blastocysts). These cells can be grown and maintained in culture thereby allowing for the introduction of the transgene via transfection and screening of the cells to identify those carrying the transgene. Transgenic ES cells are then injected into the blastocyst of a recipient embryo where they incorporate into the ICM and produce a chimeric animal (Robertson et al. 1986). The chimeras are then bred to establish a transgenic line from those embryos where the gametes were derived from the ES cells. Using ES cells enabled gene targeting as there was now the ability to transfect and screen the ES cells for the appropriate gene knockout event. However, this technique is limited to the mouse and rat (Buehr et al. 2008), the only mammalian species where true ES cells have been identified, and in chickens through the use of primordial germ cells (van de Lavoir et al. 2006).

The advent of SCNT-based cloning techniques to generate transgenic animals circumvented the need for ES cells in livestock for gene targeting. Here, the transgene can be introduced into a somatic cell in culture then fused with an enucleated oocyte (Campbell et al. 1996; Wilmut et al. 1997). The reconstituted embryo is then transplanted into a recipient animal and carried to term. As with the use of ES cells, SCNT techniques allow for precise gene modification and all the animals born will be transgenic. SCNT is now the preferred method to generate transgenic mammals with specific gene modifications.

Recent advances have also been made with transgene delivery methods. Both transposons and lentiviral vectors have the ability to efficiently integrate foreign DNA into the genome. Transposons are mobile genetic elements that use a transposase enzyme that recognizes inverted terminal repeats flanking the transposon and catalyzes the excision and relocation of the transposon to another, random site in the genome. The *Sleeping Beauty* transposon, developed from an inactive salmon transposon of the *Tc1/mariner* family (Ivics et al. 1997), has been used to generate germline transgenic mice

and rats (Dupuy et al. 2002; Carlson et al. 2010) and has the potential to be successful in livestock species. For transgenesis, pronuclear microinjection is used to introduce a plasmid containing the desired transgene inserted between the *Sleeping Beauty* inverted repeats along with the mRNA for the transposase to result in the excision of the transgene from the plasmid and into the genome producing a high percentage of germline transgenic offspring. In another approach, viral-mediated transgenesis was developed for livestock using lentiviral vectors (Whitelaw et al. 2008). Here, the transgene is inserted into a HIV-based retroviral vector and injected into the perivitelline space of an embryo. A high proportion of the resulting chimeras are transgenic and express the transgene.

14.2 Scientific Issues from Making Genetically Engineered Animals

Based on the production method used to generate a transgenic animal, several issues arise and must be considered when evaluating the health and well-being of the transgenic line. For instance, microinjection results in the random addition of the transgene into the genome. This implies that it is possible for an endogenous gene to be interrupted, which while documented in mice (Jaenisch 1988), so far has not been reported in transgenic livestock. While this remains a possibility, the random nature of transgene integration, coupled with the observations that less than 5% of mammalian DNA codes for proteins, suggests that the interruption of an endogenous gene will be a rare event. Never-the-less, transgenic lines resulting from microinjection should be carefully screened for any detrimental effects of transgene presence by both identifying the transgene insertion site and monitoring the animals for any ill effects. Microinjection is a very inefficient process in livestock species where only 1–5 % of the injected embryos integrate the transgene to result in a transgenic animal (Maga et al. 2003). In addition, it is possible that the transgene integrates into an inactive region of chromatin that will not allow for expression of the transgene product. Furthermore, if the transgene integrates after the one cell stage the result will be a mosaic animal. Due to the nature of transgene insertion, microinjection only allows for gene addition.

The potential position effects caused by random transgene insertion via pronuclear microinjection can be avoided by using cell-based methods of transgenesis such as SCNT. Here, the desired gene insertion event can be screened for prior to regenerating the embryo. This allows for very subtle and more specific modifications to be made such as the correction of a mutation or the knocking out of a disease-causing allele. However, the primary clones generated often suffer from congenital defects due to the nature of the reprogramming process whereby the differentiated somatic cell used to supply the genetic material has to revert to an embryonic state to allow for proper embryo development. This results in inefficiencies as losses occur during reconstruction, gestation and after parturition (Wells 2005). Like pronuclear

microinjection, the overall efficiency of the process is very low with approximately 0.05–1.2 % reconstructed embryos becoming a transgenic founder (Keefer 2008).

While the use of transposon- and lentivral-based methods for the production of transgenic mammals offer a more efficient means of transgene introduction, these approaches also pose some issues to the animal. For instance, resulting founders from the pronuclear microinjection of transposon transgenes often carry multiple insertions. This requires the out breeding of each founder to establish single transgenic lines. Like pronuclear microinjection, the insertion site of the transposon-based vector is random and therefore can cause insertional mutagenesis of endogenous genes. Different transposons have certain preferences for genomic insertion sites. For instance, the *Sleeping Beauty* transposon randomly integrates at TA dinucleotides distributed throughout the genome while the *piggyBac* transposon integrates at TTAA, a common sequence in transcriptional units (Clark et al. 2007). Therefore, choice of vector is critical as *piggyBac* tends to integrate near to or within coding sequences while *Sleeping Beauty* integrates into coding sequence at a rate no greater than expected by random integration (Clark et al. 2007). Furthermore, there is the possibility of the transgene sequence 'hopping' within the genome once integrated (the transposase inverted repeats are still present), although this has yet to be reported.

The use of lentiviral vectors is tenfold more efficient at producing transgenic animals with the main gains coming from improved embryo survival rates over pronuclear microinjection and SCNT (Whitelaw et al. 2004). However, there are limits on transgene size due to vector design and viral particle production can be tricky. More importantly in terms of the resulting animal, is the fact that like the use of transposons, multiple insertion sites occur requiring breeding to generate individual transgenic lines. Also, there have been reports in human cells that the lentiviruses tend to integrate within or next to genes (Clark et al. 2007), thereby increasing the chance of undesired insertional mutagenesis. Furthermore, the resulting transgenic animal will have viral sequence present in its genome.

14.3 Applications of Genetically Engineered Animals

The goal of GE is the same as breeding and selection: to introduce a desired phenotype into an animal. In the case of GE, the genetic change is introduced in the form of the transgene which is designed to express or knock-out expression of a specific protein often in a tissue- and temporally-specific fashion to result in the desired phenotype. The first demonstration of an altered phenotype in an animal via transgenesis came in 1982 when increased growth was reported in transgenic mice expressing a rat growth hormone (GH) transgene (Palmiter et al. 1982). The first reports of GE livestock soon followed in 1985 (Hammer et al. 1985). The use of GE food animals has been focused on two main areas, namely the development of improved animals or animal food products and specialized non-agricultural purposes such as using the animals as a bioreactor to produce human pharmaceuticals or compatible organs for human transplant and more recently on their use as medical models.

14.4 Genetically Engineered Animals as Biomedical Research Models

GE mice are regularly produced as biomedical research models. The first gene knocked out in the mouse was HPRT in order to establish a model of Leisch-Nyan Syndrome (Kuehn et al. 1987). However, as seen with this initial model, the mouse is not always a good model for humans and increasingly livestock models are being sought to allow study of complex human diseases (Hunter et al. 2005). To date, the best GE livestock model is the pig (Aigner et al. 2010). GE pig models of retinitis pigmentosa (Petters et al. 1997), Huntington's disease (Uchida et al. 2001), cardiovascular disease (Hao et al. 2006) and more recently cystic fibrosis (Rogers et al. 2008), Alzheimer's disease (Kragh et al. 2009) and diabetes (Umeyama et al. 2009; Renner et al. 2010) have all been reported. While regulation of these GE animals from the point of health and welfare falls under the research and Institutional Animal Care and Use Committees guidelines, they also fall under the jurisdiction of the United States Food and Drug Administration (FDA) as outlined in the guidance document released in January 2009 (Draft Guidance for Industry #187).

14.5 Genetically Engineered Animals as Bioreactors

Dairy animals and chickens both offer an attractive platform for the production of rare human pharmaceuticals. The mammary gland and the chicken tissues responsible for producing the egg white are highly synthetic tissues capable of producing large amounts of protein (23 g protein/kg body weight/day in the lactating cow and 3 g protein/egg in the chicken) that is easily attainable via milking or egg collection. Therefore, the desired drug can be manufactured by the animal in the mammary gland or oviduct and then purified from the milk or egg white. In addition to the protein synthetic capability, the drug would be produced in a vertebrate-based system and therefore undergo more appropriate post-translational modifications than can be offered by bacterial or yeast cell-culture based systems of drug production. Furthermore, once established, the drug-producing animals are easier to maintain than cell culture vat systems and require considerably less capital investment in equipment and infrastructure.

The first (and to date only) transgenic animal product approved for use is antithrombin (ATryn®) produced in the milk of transgenic goats by GTC Biotherapeutics to treat deep vein thrombosis (Ebert et al. 1991; Edmunds et al. 1998). In addition to pharmaceutical production, several other applications using the animal as a bioreactor exist and are in various stages of development. These include the production of human polyclonal antibodies in the blood of transgenic cows by the company Hematech to treat bacterial infections or immuno-deficient patients (Kuroiwa et al. 2009), the production of biologically active and correctly glycosylated human interferon α-2b in the egg whites of transgenic hens for use as a human biopharmaceutical to treat hepatitis

C (Rapp et al. 2003), development of the organophosphorous scavenger protein human butyrylcholinesterase (rBChE) made in the milk of transgenic goats by the company PharmAthene for use as a prophylactic against acute organophosphorous nerve agent toxicity (Huang et al. 2007) and the production of spider silk, also in the milk of transgenic goats, for use as bio-matrices and sutures (Williams 2003).

One other use of transgenic animals for medical purposes is the GE of animals to supply compatible tissues and organs for use in human transplants, or xenotransplantation. To meet the rising demand for replacement tissues and organs several companies, including Revivicor, have been specializing in the GE of pig tissues and organs for use in regenerative medicine. The pig is an ideal donor for humans based on the size of their organs and blood vessels. However, pig tissue is coated with a sugar (α(1,3)-galactose) that causes hyper-acute rejection of the tissue when placed into a human. The gene responsible for forming this sugar, α(1,3)-galactosyl transferase, has been disrupted in pigs using successive rounds of SCNT to achieve a double knockout of the target gene and elimination of the Gal α1,3 sugars on the organ surface (Phelps et al. 2003). Without these sugars, humans no longer see the organ as foreign and work is now proceeding by the generation of multi-transgenic pigs to address the issue of vascular rejection (Tai et al. 2007).

14.6 Genetic Engineering for Improved Animals and Products

The applications of GE animals for agriculture can be classified in four main areas based on their area of desired benefit, namely growth, environment, disease resistance, and improved animal food products. Early efforts in the area of GE food animals focused on enhancing growth. In both farmed pigs and fish, reaching market weight is an important parameter for the producer. Based on the early work in mice, transgenic salmon (Du et al. 1992) and pigs (Hammer et al. 1985) were generated that expressed GH systemically. The company AquaBounty has been developing a line of GE growth hormone Atlantic salmon named AquAdvantage® that reach market size twice as fast and convert feed into body mass 10–30 % more efficiently than traditional farmed salmon. Here, the systemic expression of GH had no detrimental effects and the final mature weight of the GE fish was not different from their non-transgenic counterparts. These GE salmon are currently under review by the FDA for approval for human consumption. If successful, these fish will represent the first GE animal food product available for consumption.

The systemic expression of GH in transgenic pigs did result in faster growth, as well as increased insulin-like growth factor (IGF) levels and feed efficiency, and less body fat. However, the pigs also suffered from a variety of health problems including lameness, ulcers and impaired reproduction, most likely due to the systemic nature of GH expression (Pursel et al. 1990). In a second attempt to increase growth, expression of human IGF was restricted to the muscle of the pigs using an avian skeletal actin promoter (Pursel et al. 2004). In this application, the animals were healthy but there was no major impact on growth. Instead, more subtle changes

occurred, with the GE pigs having less body fat and more lean tissue over time than their non-transgenic counterparts.

In another approach to increase growth in the pig, researchers at the University of Illinois focused on increasing milk production in the lactating sow. As milk production accounts for 44 % of the pre-weaning growth in pigs, they introduced a gene encoding bovine α-lactalbumin (Bleck et al. 1998), the milk protein responsible for the formation of lactose, the main sugar in milk. Lactose acts as an osmoregulator, thus the more lactose present, the more water drawn into the mammary gland and the more milk that is made. Transgenic pigs over-expressing α-lactalbumin made more milk from days 3–9 of lactation and transgenic-reared litters gained weight at a higher rate than control-reared litters (Noble et al. 2002). The same group also expressed human IGF-I in the mammary gland and while milk yield and litter weight gain was not different, intestinal mucosal weight and enzyme activity was increased (Monaco et al. 2005). These various uses of GE to increase growth in farmed animals are another approach to maintaining the sustainability of large-scale protein supply for human food production.

GE has also been used to address the issue of environmental pollution and the sustainability of farming. The digestive system of monogastric animals such as pigs and chickens results in a high phosphorus content of their manure. Phytate is the most abundant source of plant-derived phosphorous in the diet of these animals coming from cereal grains and oil seeds. However, pigs and chickens cannot digest the natural phytate present in their feed and require inorganic phosphate supplements. The undigestible phosphate in the form of phytate is then passed undigested into the feces and is the single most important manure-derived pollutant in the pork industry. Researchers at the University of Guelph in Canada have produced transgenic pigs that express the *E. coli* phytase gene in the salivary gland under control of the mouse parotid secretory protein promoter (Golovan et al. 2001). The presence of phytase in the saliva allows the animals to digest the phosphate in the dietary phytate and fecal phosphorus output in these transgenic pigs is reduced by 75 %. This represents a significant beneficial impact on the environment.

Animals are also being GE to address disease and welfare problems associated with animals. Mastitis, or infection of the udder, is a common and costly disease in the dairy industry both in terms of economics losses from decreased production and cost of treatment, and animal well-being, as many infections cannot be cured with antibiotics and result in the culling of the animal. Researchers at the USDA-ARS generated transgenic dairy cattle by SCNT that express the bacterial-derived enzyme lysostaphin in their milk (Wall et al. 2005). Lysostaphin specifically degrades *Staphylococcus aureus*, one of the main and hard to treat mastitis-causing pathogens. Upon challenge with *S. aureus*, 71 % of control glands became infected compared to only 14 % of transgenic glands. This represents a major advance in the treatment and prevention of *S. aureus* mastitis.

Another disease issue being addressed in dairy cattle via GE is bovine spongiform encephalopathy (BSE) or 'mad cow disease'. This is a transmissible neurodegenerative disease caused by misfolded prion proteins. To mitigate the risks associated with this prion, the PRNP gene locus that encodes the prion protein was knocked out in cattle using SCNT techniques (Richt et al. 2007). These cattle appear

healthy and resist prion infection *in vitro*. The disruption of this gene could reduce the risk of other cows and humans contracting the disease.

GE is also being used to generate animal food products with components that enhance the nutritive value or safety of the food and thereby improve human health. Animal food products are often high in saturated fats, consumption of which can raise serum cholesterol levels leading to a variety of health problems. GE has been used to lower the levels of these human unhealthy fats in both milk and meat. At the University of California, Davis, transgenic goats expressing rat stearoyl-CoA desaturase in the mammary gland were generated by pronuclear microinjection (Reh et al. 2004). Desaturase enzymes act to add double bonds to saturated fatty acids, thereby producing more heart-healthy mono- and polyunsaturated fats. While the total amount of fat in the milk remained the same, milk from transgenic goats had lower levels of saturated fats and higher levels of mono- and polyunsaturated fat, indicating the desired function of the transgene. Fat composition in meat has also been modified using the transgenic approach by the systemic expression of the *fat-1* gene of *C. elegans* in pigs (Lai et al. 2006). Mammals lack this key gene that can convert unsaturated fat substrates into omega-3 fatty acids. Expression of *fat-1* resulted in increased levels of omega-3 fatty acids in the organs and tissues (including meat) of the transgenic pigs.

To increase the protein content of milk, GE cows were generated that contained additional copies of two main milk proteins, β- and κ–casein (Brophy et al. 2003). While the transgenic animals did make more κ-casein, they did so at the expense of other proteins. The milk of transgenic animals also had a distinct color change from white to yellow (likely the result of a disrupted micelle structure) and the amount of fat and minerals was also altered. While not achieving the desired results, insight has been gained into protein production in the mammary gland. In another attempt to improve the composition of milk, researchers at the University of California, Davis have produced transgenic dairy goats expressing human lysozyme in their milk (Maga et al. 2003, 2006a). Lysozyme is an antimicrobial protein normally found in human milk whose presence in livestock milk has been shown to mimic human milk by slowing the growth of bacterial pathogens *in vitro* (Maga et al. 2006b) and positively impacting the intestine upon consumption by model animals (Brundige et al. 2008, 2010). Data collected over the years on most applications of transgenic animals for agriculture indicate that the implementation of GE animals can indeed have a positive impact on animal productivity and sustainability and human and animal health.

14.7 Regulatory Issues from Applications

In assessing the safety of transgenic animals and their products, each transgenic line should be considered separately, based on the application and method of generation, until such time as a sufficient body of data has been accumulated to allow discrimination of what aspects of GE production or product type need specific regulatory

attention and which pose an insignificant risk. For instance, possible insertion site consequences must be determined if random transgene integration events occurred. This can be accomplished by mapping the site of integration using molecular techniques. In animals, transgene stability is assessed by breeding to determine if the transgene segregates in a Mendelian fashion. Off-target or indirect effects of the presence of transgene itself as well as the transgene product must also be considered. This is not as straight forward as the determination of integration events and transgene segregation. If there is a major negative impact of the transgene and its expression, this may be manifested as a phenotype easy to spot such as the ailments with the growth hormone transgenic pigs (Hammer et al. 1985) and sheep (Nancarrow et al. 1991). However, minor effects may be difficult if not impossible to prove. Assessment of the well-being of transgenic lines can be made in the broad sense by studying the basic functions of the animals, such as reproduction, growth, lactation and behavior, compared to non-transgenic sibs and applying published standards for the species in question as done by Jackson et al. (2010) for the human lysozyme transgenic goats. More subtle effects may be detected using further molecular and metabolite analyses as warranted by the application.

The safety of the transgene product also needs to be determined on a case by case basis. In terms of pharmaceuticals or compounds isolated from the transgenic animal for other uses, the GE-derived product must show equivalence to the market standard. In terms of the animals themselves being the transgenic product (i.e. GH salmon, lysozyme milk, pork with more omega-3 fats), the composition of the final product as well as the function of the transgene product if ingested must be considered.

14.8 Regulatory Guidelines for Genetically Engineered Animals

The framework for the regulation of GE food animals and their products in the US and Canada is based on guidelines set forth by Codex. The Codex Alimentarius Commission was established jointly in 1963 by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) to develop food standards and guidelines to ensure consumer health and fair trade of food. The standards are science-based and adopted by consensus of scientists, technical experts, government regulators and international consumer and industry organizations. The ad hoc Codex International Task Force on Foods Derived from Biotechnology was established in 1999 to address the safety, consumer health and nutritional implications of foods derived from biotechnology based on scientific evidence and risk analysis, with the goal of establishing standards and guidelines for the assessment of the safety and implementation of GE foods. The Task Force completed their work and was dissolved in 2008 with the release of guidelines for both the risk analysis (CAC/GL 44-2003- Principles for the Risk Analysis of Food Derived from Modern Biotechnology) and food safety assessment (CAC/GL 68-2008- Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Animals) of GE foods.

These two documents offer an approach to assess any hazards and safety issues associated with food derived by GE. They are based on the premise that the safety of foods derived from GE animals be characterized with respect to conventional counterparts, considering both intended and unintended effects of the transgene. As most food has not been scientifically studied in a fashion that would characterize ***ALL*** risks associated with the particular food product, the evaluation of GE-derived food from animals is meant to identify any new or altered hazards relative to the non-GE counterpart. The recommended approach takes into consideration the composition of the transgene and its product, the health of the GE animal, and the composition of the food product from the animal. If any changes (hazards) are identified by the safety assessment, the risk assessment component would then identify the risk or relevance of the change to human health. Assessment must be done on a case-by case basis and the process involves the collection of scientific data from both the developer of GE product and independent scientists, the scientific literature, regulatory agencies and other interested parties.

In the United States, the Center for Veterinary Medicine (CVM) of the FDA is the regulatory authority for GE animals under the New Animal Drug provisions of the Federal Food Drug and Cosmetic Act (FFDCA). Under the FFDCA, the presence of a transgene in an animal intended to impact the structure or function of the animal, regardless of the use of the product derived from the animal, has been deemed a new drug and thus applicable to the New Animal Drug Application (NADA) or Investigational New Animal Drug (INAD) approval process. In developing their regulatory framework for GE animals, the FDA considered the Codex recommendations and public input. A draft guidance on the regulation of GE animals containing a heritable rDNA construct was issued by the FDA in September 2008 (Draft Guidance for Industry #187), followed by a 60 day period where public comment was allowed. The final guidance was issued in January 2009 and took into account the 28,000 public comments received.

The FDA guidance states that the NADA requires demonstration of seven components, six of which regard the identity of the animals and safety issues and one regarding the claim of the product. The first component involves the identification of the GE event by indicating species and intended use of the animal and/or food product (name and function of the transgene), the ploidy and zygosity of the transgene and by providing molecular data identifying the number of integration events and insertion site of the transgene. The second component requires data on the molecular characterization of the transgene in terms of the source of DNA used in the transgene construct, the sequence of the transgene, details on transgene assembly and purity of the transgene construct prior to introduction. This information is required in order to make an assessment whether or not the DNA sequences present have the potential to encode toxins, allergens or pathogens or could dysregulate cellular function. The third component requires information on the method of introduction of the transgene and breeding strategy to maintain the line. Next, the health of the GE animal must be documented by providing veterinary and treatment records, and data on general production parameters such as growth, reproduction, and behavior as well as the physiology (blood work etc.) of the transgenic line.

The fifth component requires demonstration of the stable inheritance of the integrated transgene and stable expression of the transgene product. The sixth component focuses on food safety (any direct toxicity and unintended effects) as outlined by Codex and also considers environmental safety. The seventh and final component addresses the validation of claim, in other words, will the transgene, and thus the GE product, function as claimed in the application.

Once reviewed by the FDA, data is delivered to a Veterinary Medicine Advisory Committee (VMAC) for evaluation, whose traditional function has been to review the FDA's analysis of the data presented in an application for approval of a drug. The VMAC is composed of a standing panel of experts in animal and human health as well as invited members with expertise in the particular field under consideration. The VMAC makes regulatory recommendations to the Commissioner of the FDA on the safety and effectiveness claims of the product. One GE animal product has been approved by this process, ATryn®, a pharmaceutical produced in the milk of GE goats. Several other applications are pending and there has been a recommendation on another (GH salmon). It should be noted that the VMAC provides advice only for what is asked for in the application.

14.9 Issues Confronting the Implementation of Genetically Engineered Animals

As we have noted before (Murray and Maga 2010), the debate about the introduction of GE animals into the food supply usually raises the same list of risks or concerns, including animal welfare, loss of genetic diversity, food safety, environmental release, and un-intended consequences such as activation of quiescent viruses or inappropriate gene expression resulting from activation of endogenous genes, all of which are addressed by the US regulatory framework under the auspices of the FDA. However, it should be noted that the concerns about animal welfare and loss of genetic diversity are general issues for all domesticated food animals and, as such, are not unique to the production and use of GE animals. The loss of genetic diversity is a function of selective breeding coupled with economic considerations, and is not unique to GE, nor caused directly by being GE.

Food safety issues are assessed on a case-by-case basis according to protocols developed to determine the potential risk of a new protein (FAO/WHO 2008), as new proteins may be allergenic or toxic. While plants contain many toxic compounds, whose expression and therefore concentration could potentially be affected by the integration of a transgene, agriculturally important mammals and poultry do not naturally express toxins. However, some fish do express toxins and many animals express proteins that are allergens for some individuals. The expression of the genes encoding these proteins could potentially be altered by transgene integration or expression, as well as by mutations, although the probability is slight.

The risk of environmental damage due to the release of GE animals, particularly fish, was noted in one report (NRC 2002) as perhaps the most significant risk associated with

GE food animals. These risks will need to be assessed on a case-by-case basis with consideration given to the species involved, the specific nature of the transgene construct used and its resulting product, and the environment in the area where release might occur. This is addressed in the FDA Guidance document (Draft Guidance for Industry #187) in the requirement for an environmental assessment and, if necessary, a full environmental impact statement.

Finally, perhaps the most difficult risks to assess are those labeled as "un-intended consequences", as they are non-specific. Two observations help to put this risk category into perspective. The first being the observation that throughout the course of human evolution people have eaten virtually all developmental stages of most common animals, birds and fish. This means we have been exposed to all the products from the genes in these animals, including quiescent viral sequences. Second, horizontal gene transfer in the absence of transposable elements or viral vectors is exceedingly rare, if it occurs at all, in higher eukaryotes. We do not generally take up and integrate functional genes from the DNA contained in the food we consume each day, including that of the accompanying bacteria and viruses. Combined, these observations suggest that the risk of a significantly negative adverse effect from the use of GE animals for food is fairly unlikely, particularly with the requirements for premarket approval, which among other things assesses the likelihood that the transgene product may be toxic or allergenic as noted above.

However, one concept missing from this debate is the cost of **not** using transgenic technology to benefit agriculture; that is, the positive components of the risk benefit analysis (Murray and Maga 2010). All of the above risks of using GE animals have been thoroughly discussed and examined, and the possibility of harmful effects are addressed in the regulatory framework. However, the risks, or cost, to human welfare from not using GE livestock, poultry and fish has yet to be addressed. There are areas where a GE approach can contribute to solving a specific problem where the more traditional approaches of selective breeding and management cannot. GE of an animal is not carried out in isolation from other approaches used in animal agriculture, but rather is used in conjunction with selective breeding, veterinary intervention, nutrition, and animal management techniques. As seen by the GE livestock applications currently in progress, GE can make a positive impact on the quantity and quality of the food supply. After almost 30 years of research, several applications of GE animals are awaiting an approval decision. These decisions will no doubt greatly impact the future of this field.

References

Aigner B, Renner S, Kessler B, Klymiuk N, Kurome M, Wünsch A, Wolf E (2010) Transgenic pigs as models for translational biomedical research. J Mol Med 88:653–664

Bleck GT, White BR, Miller DJ, Wheeler MB (1998) Production of bovine α-lactalbumin in the milk of transgenic pigs. J Anim Sci 76:3072–3078

Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P, Laible G (2003) Cloned transgenic cattle produce milk with higher levels of β-casein and κ-casein. Nat Biotechnol 21:157–162

Brundige DR, Maga EA, Klasing KC, Murray JD (2008) Lysozyme transgenic goats' milk influences gastrointestinal morphology in young pigs. J Nutr 138:921–926

Brundige DR, Maga EA, Klasing KC, Murray JD (2010) Consumption of pasteurized human lysozyme transgenic goats' milk alters serum metabolite profile in young pigs. Transgenic Res 19:563–574

Buehr M, Meek S, Blair K, Yang J, Ure J, Silva J, McLay R, Hall J, Ying QL, Smith A (2008) Capture of authentic embryonic stem cells from rat blastocysts. Cell 135:1287–1298

CAC/GL 44–2003. Principles for the risk analysis of food derived from modern biotechnology. http://www.codexalimentarius.net/web/more_info.jsp?id_sta=10007. Accessed 27 Dec 2011

CAC/GL 68–2008. Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA animals. http://www.codexalimentarius.net/web/more_info.jsp?id_sta=11023. Accessed 27 Dec 2011

Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line. Nature 380:64–68

Carlson DF, Geurts AM, Garbe JR, Park CW, Rangel-Filho A, O'Grady SM, Jacob HJ, Steer CJ, Largaespada DA, Fahrenkrug SC (2010) Efficient mammalian germline transgenesis by cis-enhanced Sleeping Beauty transposition. Transgenic Res 20:29–45. doi:10.1007/s11248-010-9386-5

Clark KJ, Carlson DF, Fahrenkrug SC (2007) Pigs taking wing with transposons and recombinases. Genome Biol 8(Suppl 1):S13.1–S13.16

Draft Guidance for Industry #187: Regulation of genetically engineered animals containing heritable rDNA constructs. http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM113903.pdf. Accessed 27 Dec 2011

Du SJ, Gong Z, Fletcher G, Shears M, King MJ, Idler DR, Hew CL (1992) Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. Biotechnology 10:176–181

Dupuy AJ, Clark K, Carlson CM, Fritz S, Davidson AE, Markley KM, Finley K, Fletcher CF, Ekker SC, Hackett PB, Horn S, Largaespada DA (2002) Mammalian germline transgenesis by transposition. Proc Natl Acad Sci USA 99:4495–4499

Ebert KM, Selgrath JP, DiTullio P, Denman J, Smith TE, Memon MA, Schindler JE, Monastersky GM, Vitale JA, Gordon K (1991) Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. Biotechnology 9:835–838

Edmunds T, Van Patten SM, Pollock J, Hanson E, Bernasconi R, Higgins E, Manavalan P, Ziomek C, Meade H, McPherson JM, Cole ES (1998) Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin. Blood 91:4561–4571

Joint FAO/WHO Food Standards Programme (2008) Codex Alimentarius Commission: Report of the Thirty-First Session, Ref. No. ALINORM 08/31/REP 2008, FAO, Rome

Golovan SP, Meidinger RG, Ajakaiye A, Cottrill M, Wiederkehr MZ, Barney DJ, Plante C, Pollard JW, Fan MZ, Hayes MA, Laursen J, Hjorth JP, Hacker RR, Phillips JP, Forsberg CW (2001) Pigs expressing salivary phytase produce low-phosphorus manure. Nat Biotechnol 19:741–745

Gordon JW, Ruddle FH (1981) Integration and stable germ line transmission of genes injected into mouse pronuclei. Science 21:1244–1246

Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683

Hao YH, Yong HY, Murphy CN, Wax D, Samuel M, Rieke A, Lai L, Liu Z, Durtschi DC, Welbern VR, Price EM, McAllister RM, Turk JR, Laughlin MH, Prather RS, Rucker EB (2006) Production of endothelial nitric oxide synthase (eNOS) over-expressing piglets. Transgenic Res 15:739–750

Huang YJ, Huang Y, Baldassarre H, Wang B, Lazaris A, Leduc M, Bilodeau AS, Bellemare A, Côté M, Herskovits P, Touati M, Turcotte C, Valeanu L, Lemée N, Wilgus H, Bégin I, Bhatia B, Rao K, Neveu N, Brochu E, Pierson J, Hockley DK, Cerasoli DM, Lenz DE,

Karatzas CN, Langermann S (2007) Recombinant human butyrylcholinesterase from milk of transgenic animals to protect against organophosphate poisoning. Proc Natl Acad Sci USA 104:13603–13608

Hunter CV, Tiley LS, Sang HM (2005) Developments in transgenic technology: applications for medicine. Trends Mol Med 11:293–298

Ivics Z, Hackett PB, Plasterk RH, Izsvák Z (1997) Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. Cell 91:501–510

Jackson KA, Berg JM, Murray JD, Maga EA (2010) Evaluating the fitness of human lysozyme transgenic dairy goats: growth and reproductive traits. Trans Res 19:977–986

Jaenisch R (1988) Transgenic animals. Science 240:1468–1474

Keefer CL (2008) Lessons learned from nuclear transfer (cloning). Theriogenology 69:48–54

Kragh PM, Nielsen AL, Li J, Du Y, Lin L, Schmidt M, Bogh IB, Holm IE, Jakobsen JE, Johansen MG, Purup S, Bolund L, Vajta G, Jørgensen AL (2009) Hemizygous minipigs produced by random gene insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APPsw. Transgenic Res 18:545–558

Kuehn MR, Bradley A, Robertson EJ, Evans MJ (1987) A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. Nature 326:295–298

Kuroiwa Y, Kasinathan P, Sathiyaseelan T, Jiao JA, Matsushita H, Sathiyaseelan J, Wu H, Mellquist J, Hammitt M, Koster J, Kamoda S, Tachibana K, Ishida I, Robl JM (2009) Antigen-specific human polyclonal antibodies from hyperimmunized cattle. Nat Biotechnol 27:173–181

Lai L, Kang JX, Li R, Wang J, Witt WT, Yong HY, Hao Y, Wax DM, Murphy CN, Rieke A, Samuel M, Linville ML, Korte SW, Evans RW, Starzl TE, Prather RS, Dai Y (2006) Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat Biotechnol 24:435–436

Maga EA, Sargent RG, Zeng H, Pati S, Zarling DA, Oppenheim SM, Collette NMB, Moyer AL, Conrad-Brink JS, Rowe JD, BonDurant RH, Anderson GB, Murray JD (2003) Increased efficiency of transgenic livestock production. Transgenic Res 12:485–496

Maga EA, Shoemaker CF, Rowe JD, BonDurant RH, Anderson GB, Murray JD (2006a) Production and processing of milk from transgenic goats expressing human lysozyme in the mammary gland. J Dairy Sci 89:518–524

Maga EA, Cullor JS, Smith W, Anderson GB, Murray JD (2006b) Human lysozyme expressed in the mammary gland of transgenic dairy goats can inhibit the growth of bacteria that cause mastitis and the cold-spoilage of milk. Foodborne Pathog Dis 3:384–392

Monaco MH, Gronlund DE, Bleck GT, Hurley WL, Wheeler MB, Donovan SM (2005) Mammary specific transgenic over-expression of insulin-like growth factor-I (IGF-I) increases pig milk IGF-I and IGF binding proteins, with no effect on milk composition or yield. Transgenic Res 14:761–773

Murray JD, Maga EA (2010) Is there a risk from not using GE animals? Transgenic Res 19:357–361

Nancarrow CD, Marshall JTA, Clarkson JL, Murray JD, Millard RM, Shanahan CM, Wynn PC, Ward KA (1991) Expression and physiology of performance regulating genes in transgenic sheep. J Reprod Fertil Suppl 43:277–291

Noble MS, Rodriguez-Zas S, Cook JB, Bleck GT, Hurley WL, Wheeler MB (2002) Lactational performance of first parity transgenic gilts expressing bovine α-lactalbumin in their milk. J Anim Sci 80:1090–1096

NRC (2002) Animal biotechnology: science based concerns. National Academies Press, Washington, DC

Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300:611–615

Petters RM, Alexander CA, Wells KD, Collins EB, Sommer JR, Blanton MR, Rojas G, Hao Y, Flowers WL, Banin E, Cideciyan AV, Jacobson SG, Wong F (1997) Genetically engineered large animal model for studying cone photoreceptor survival and degeneration in retinitis pigmentosa. Nat Biotechnol 15:965–970

Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y, Ayares DL (2003) Production of alpha 1,3-galactosyltransferase-deficient pigs. Science 299:411–414

Pursel VG, Bolt DJ, Miller KF, Pinkert CA, Hammer RE, Palmiter RD, Brinster RL (1990) Expression and performance in transgenic pigs. J Reprod Fertil Suppl 40:235–245

Pursel VG, Mitchell AD, Bee G, Elsasser TH, McMurtry JP, Wall RJ, Coleman ME, Schwartz RJ (2004) Growth and tissue accretion rates of swine expressing an insulin-like growth factor I transgene. Anim Biotechnol 15:33–45

Rapp JC, Harvey AJ, Speksnijder GL, Hu W, Ivarie R (2003) Biologically active human interferon alpha-2b produced in the egg white of transgenic hens. Transgenic Res 12:569–575

Reh WA, Maga EA, Collette NMB, Moyer A, Conrad-Brink JS, Taylor SJ, DePeters EJ, Oppenheim S, Rowe JD, BonDurant RH, Anderson GB, Murray JD (2004) Using a stearoyl-CoA desaturase transgene to alter milk fatty acid composition. J Dairy Sci 87:3510–3514

Renner S, Fehlings C, Herbach N, Hofmann A, von Waldthausen DC, Kessler B, Ulrichs K, Chodnevskaja I, Moskalenko V, Amselgruber W, Göke B, Pfeifer A, Wanke R, Wolf E (2010) Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired GIP function. Diabetes 59:1228–1238

Richt JA, Kasinathan P, Hamir AN, Castilla J, Sathiyaseelan T, Vargas F, Sathiyaseelan J, Wu H, Matsushita H, Koster J, Kato S, Ishida I, Soto C, Robl JM, Kuroiwa Y (2007) Production of cattle lacking prion protein. Nat Biotechnol 25:132–138

Robertson E, Bradley A, Kuehn M, Evans M (1986) Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323:445–448

Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB Jr, Zabner J, Prather RS, Welsh MJ (2008) Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science 321:1837–1841

Tai HC, Ezzelarab M, Hara H, Ayares D, Cooper DK (2007) Progress in xenotransplantation following the introduction of gene-knockout technology. Transpl Int 20:107–117

Uchida M, Shimatsu Y, Onoe K, Matsuyama N, Niki R, Ikeda JE, Imai H (2001) Production of transgenic miniature pigs by pronuclear microinjection. Transgenic Res 10:577–582

Umeyama K, Watanabe M, Saito H, Kurome M, Tohi S, Matsunari H, Miki K, Nagashima H (2009) Dominant-negative mutant hepatocyte nuclear factor 1alpha induces diabetes in transgenic-cloned pigs. Transgenic Res 18:697–706

van de Lavoir MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessaro TM, Swanberg SE, Delany ME, Etches RJ (2006) Germline transmission of genetically modified primordial germ cells. Nature 441:766–769

Wall RJ, Powell AM, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, Wells KD, Talbot N, Hawk HW (2005) Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. Nat Biotechnol 23:445–451

Wells DN (2005) Animal cloning: problems and prospects. Rev Sci Tech 24:251–264

Whitelaw CB, Radcliffe PA, Ritchie WA, Carlisle A, Ellard FM, Pena RN, Rowe J, Clark AJ, King TJ, Mitrophanous KA (2004) Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector. FEBS Lett 571:233–236

Whitelaw CBA, Lilico SG, King T (2008) Production of transgenic farm animals by viral vector-mediated gene transfer. Reprod Domest Anim 43(Suppl 2):355–358

Williams D (2003) Sows' ears, silk purses and goats' milk: new production methods and medical applications for silk. Med Dev Technol 14:9–11

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813

Chapter 15
Regulatory Science, Research Science and Innovation in Agricultural Biotechnology

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Abstract Regulatory science produces data needed for risk assessments that help regulators make decisions about whether to allow certain activities such as the cultivation of transgenic crops. Research science, on the other hand, seeks to further objective knowledge for its own sake. Regulatory and research science have the same structure of erecting hypotheses as tentative answers to problems, and testing, that is attempting to falsify, those hypotheses by comparing their predictions with observations. In this paper, we discuss important differences between regulatory science and research, and in particular how they differ in the formulation and testing of hypotheses: regulatory science tests hypotheses that seek categorization of effects, whereas interesting research tends to test hypotheses that make precise quantitative predictions. When regulatory science is confused with research, many irrelevant data are produced, which confuse and delay decision-making, and increase the costs of regulation to the developer and regulator, ultimately harming innovation of new technology because business risks are too high. If research is confused with regulatory science, uninteresting hypotheses are tested, which slow the development of knowledge, again harming innovation. In some cases, particularly very early in the development of new technology, regulatory science and research may be

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indistinguishable; however, it is important for the effective development of new technology that regulatory data requirements are not laid down to answer research questions.

Keywords Biotechnology • Bucket theory • Hypothesis testing • Problem selection • Regulatory science • Risk assessment • Transgenic

15.1 Introduction

The cultivation of transgenic crops over the last 15 years has realized health, environmental and economic benefits in developed and developing countries (Brookes and Barfoot 2008, 2010; Qaim 2009; Raybould and Quemada 2010). The most widely documented benefits have come from transgenic crops with enhanced herbicide tolerance or insect resistance, but significant benefits have also resulted from virus-resistant crops (Fuchs and Gonsalves 2007). Experience with current transgenic crops suggests that agricultural biotechnology will help to solve some of the problems posed by the need to increase production of food, fuel and fiber under changing environmental conditions without worsening the loss of biodiversity (Federoff et al. 2010; Godfray et al. 2010); however, the innovation necessary for agricultural biotechnology to solve these problems may be constrained by high regulatory costs that limit research to products of interest to companies able to bear those costs (Chataway et al. 2006; Mittra et al. 2011).

Products of agricultural biotechnology that are regulated must be granted approvals by a competent authority before they can be used freely. High regulatory costs incurred by agricultural biotechnology arise from two main sources. First, large amounts of scientific data on the product and its intended use must be supplied to the competent authority for use in its deliberations on whether or not to approve the proposed use of the product. Secondly, decision-making on approvals may be lengthy and unpredictable; for example, in the European Union, some applications for commercial cultivation of transgenic crops are still awaiting decisions over 13 years after submission. Long and unpredictable regulatory decision-making complicates investment decisions and delays return on investment in the development of the product. The data and decision-making often interact to increase costs because large amounts of data complicate decision-making and data may be collected in a vain attempt to define decision-making criteria (Johnson et al. 2007; Raybould 2007).

Regulatory decision-making usually involves assessment of the risks posed by a proposed activity, such as cultivation of a particular transgenic crop, where risk is a function of the seriousness of the potential harms caused by the activity and the likelihood of those harms arising. Where significant risks are identified, evaluation of whether risk management suitably lowers risk may be considered by decision-makers. As well as risk, decision-making may include evaluation of the opportunities presented by the activity, where opportunity is a function of the size of potential benefits of the activity and the likelihood of those benefits arising. Assessing risk – and opportunity

– should be similar to fundamental research in that data are collected to test hypotheses. Risk assessment and fundamental research are different because in risk assessment hypotheses should be designed to help subjective decision-making, whereas in fundamental research, hypotheses are tested to increase objective knowledge.

In this article, we describe the importance of hypothesis testing in risk assessment and management, and why it is vital that risk assessment is not confused with fundamental research: testing no hypothesis, or unsuitable hypotheses, leads to the collection of large amounts of data that are irrelevant to risk assessment and unnecessarily constrain the invention of potentially beneficial products.

15.2 Risk Assessment as Hypothesis Testing

The philosophy of scientific discovery deals with two different methods: induction and deduction. Induction makes generalizations from particular observations and is the basis of empiricism, which proposes that objective knowledge originates from observations made without preconceptions. In an essay published in German in 1949 and in English in 1972, Karl Popper (1979) describes empiricism as the "bucket theory" of scientific knowledge: observations are accumulated in a metaphorical bucket and accrete into knowledge. Eventually there may be sufficient observations supporting a generalization that it is regarded as true.

Popper (1959, 1979) proposed an alternative theory whereby knowledge increases through observations that test our preconceptions. In this theory, observations are made in response to preconceptions; that is, we always have expectations or hypotheses that guide our observations. Hypotheses are used to deduce particular expected facts, and when our observations differ from what we expected, we formulate new hypotheses in attempts to eliminate the flaws that led to the erroneous expectations. Knowledge thereby grows by repeated testing and correction of hypotheses.

Induction has proved problematic as a logical basis for science; for example, however many facts are added to the bucket, it is never possible to prove that no subsequent observation will contradict the generalizations drawn from those facts. A second problem is that infinite generalizations can be drawn from any set of observations, and simply adding similar observations to the bucket does not help to discriminate among those generalizations. Popper (1959) offered deduction as a solution to the problem of the logic of science. He proposed that we discriminate between hypotheses by searching for experimental conditions under which the hypotheses make different predictions. Hypotheses that make accurate predictions are corroborated and survive for further testing, whereas hypotheses that make inaccurate predictions are revised or discarded. Popper argued that it is active criticism of hypotheses, not the accumulation of facts in favor of hypotheses, which advances science.

The problems of the bucket theory of science also apply to risk assessment. First, it is not possible to prove that an activity is safe, because regardless of how many times the activity has been performed safely, there is no guarantee that harmful

effects will never be observed. Secondly, there is the problem of drawing different generalizations from the same collection of facts. Sarewitz (2004) has pointed out that science often makes environmental controversies worse because disagreements are not about science but about values. Trying to settle arguments by collecting more data increases controversy because opponents have a larger collection of data from which to select facts to support their argument. Finally, risk is a function of the seriousness of the harm that may arise from an activity and the likelihood of that harm arising as a result of the activity. What society regards as a harmful effect cannot be discovered by scientific research, it must be defined by policy objectives.

Problems with the bucket theory show that a risk assessment cannot be improved simply by collecting more data. To identify useful data, it is necessary to think of a risk assessment as hypothesis testing not data gathering, or as an exercise in deductive not inductive logic. Popper (1979) gives a simple scheme to show how objective knowledge grows by deductive logic:

$$\rightarrow \text{ initial problem } [P_1] \rightarrow \text{ tentative solution } [TS] \rightarrow \text{ error elimination } [EE] \rightarrow$$
$$\text{new knowledge and a new problem } [P_2] \rightarrow$$

The initial problem is a discrepancy between a tentative solution to a previous problem and observations made to test that solution.

The scheme may be adapted to give the structure of a risk assessment for cultivation of a transgenic crop (Raybould 2006, 2010):

$$\text{Decide what constitute harmful effects of cultivating the transgenic crop } [P_1] \rightarrow$$
$$\text{hypotheses that cultivation of the crop will not cause harm } [TS] \rightarrow \text{ test the}$$
$$\text{hypotheses } [EE] \rightarrow \text{ increased knowledge of risk } [P_2] \rightarrow$$
$$\text{decision-making } [TS_2] \rightarrow$$

This simple scheme provides a conceptual framework for assessing the risks posed by the cultivation of transgenic crops (Raybould 2006; Wolt et al. 2010). The scheme could also be applied to risk management, where the hypotheses under test would be of the form “cultivation of the crop with the proposed risk management reduces the probability of harm below an acceptable threshold”. The following sections discuss how the scheme may be implemented in practice.

15.3 Formulating and Testing Risk Hypotheses

Formulation of risk hypotheses begins with a conceptual model, scenario or pathway that describes how cultivation of the transgenic crop may cause harm. As far as practicable, this procedure should start by defining harmful effects from policy objectives, regulations or other guidance, and then analyze how cultivation of the crop could bring about those effects. Working out all possible effects

of cultivating a transgenic crop, and trying to deduce which are harmful is inefficient and ineffective.

In a conceptual model, the links in the chain of events from cultivation to harmful effects are logical: what are the necessary conditions for harm to arise, not what is the likelihood of those conditions occurring. There may be infinite ways by which harm *could* arise, it is necessary, therefore, to reduce the number of scenarios that will be used to generate hypotheses for testing in the risk assessment. Some logically possible scenarios may appear so implausible that it is almost inconceivable that they pose any risk, and therefore they are not evaluated in the risk assessment. It is important to recognize and explain that implausibility means that at least one step in the scenario is known to be highly unlikely; that is, if event A is necessary for harm to arise, existing data corroborate with extremely high confidence the hypothesis that event A does not occur (Raybould 2011).

The remaining plausible scenarios are the source of the risk hypotheses tested in the risk assessment. These scenarios may be examined in terms of discrete steps that must occur for the cultivation of the transgenic crop to result in harmful effects. From each step it is possible to formulate a hypothesis, which if corroborated or falsified by suitable testing, would characterize risk in a form that is useful to decision-makers. Hypotheses could take several forms: event A does not lead to event B; event A leads to event B at a frequency below that which would cause harm; or event A leads to event B, but event B is below the magnitude necessary for harm (Raybould 2006, 2010). In each case, the hypothesis can be regarded as a hypothesis of no harm from cultivation of the transgenic crop. Testing hypotheses of no harm, with new studies, with existing data collected for other purposes independently of the current risk assessment, or both, is the basis of risk characterization.

Initial tests of risk hypotheses are made under conservative conditions designed to minimize false negatives; in other words, if the hypothesis is that "event A will not occur", tests are made under conditions most likely to reveal the potential for A to occur. Two examples illustrate the point. First, if event A is adverse effects of an insecticidal protein on a group of non-target organisms, a conservative test is exposure, in the laboratory, of suitable representative test species to the protein at ten times the highest exposure likely to result from cultivation of the transgenic crop (Raybould et al. 2007, 2011a; Romeis et al. 2008; Raybould and Vlachos 2011). If no adverse effects are seen at this concentration, experiments using exposures to the protein at field concentrations add little to the risk assessment because the test is less likely to detect adverse effects (Raybould 2006). Should adverse effects of the protein be detected in the laboratory, further studies under more realistic conditions may be conducted to evaluate whether toxicity of the protein is likely to result in harmful effects in the field. Secondly, if event A is hybridization between a crop and a wild plant species, a conservative test is artificial cross-pollination of the species in the laboratory followed by embryo-rescue to detect any hybrid seed. If hybrids are not detected under these conditions, testing could stop; if hybridization is detected, the potential for hybridization in the field could be assessed, for example, by allowing the species to cross-pollinate spontaneously under glasshouse conditions (Raybould and Cooper 2005).

The concept of starting with conservative tests most likely to reveal the potential for harm and only moving to more realistic tests if that potential is detected is called tiered testing (Touart and Maciorowski 1997; Garcia-Alonso et al. 2006). It is an effective way efficiently to characterize activities into those that pose low risk and require little or no further evaluation, and those that may pose high risk and require further assessment to determine the level of risk. The criterion for deciding whether further testing is required is a judgment about the best balance between the costs of over-testing some activities that pose low risk and the costs of incorrectly determining that high risk activities pose low risk (Chapman et al. 1998; Caley et al. 2006).

Evaluation of risk management plans follows a similar conceptual framework to risk assessment in that hypotheses about the likelihood of harm following an action are tested. In risk assessment, the scenarios might start with unrestricted cultivation of the transgenic crop. In risk management, scenarios are developed from cultivation of the transgenic crop along with measures to limit the likelihood of harm arising. The evolution in pests of resistance to insecticidal proteins is regarded as a harmful effect of cultivating transgenic insect-resistant crops (e.g., McGaughey and Whalon 1992; McGaughey et al. 1998), and in many countries, suitable insect resistance management (IRM) plans are mandatory for regulatory approvals of such crops (MacIntosh 2010).

Current IRM plans originate from a high-dose – refuge strategy for the first commercial transgenic crops resistant to lepidopterous pests, which assumed, among other things, that resistance to the insecticidal protein is controlled by a single gene, and that alleles conferring resistance are recessive and rare, and therefore almost all resistance alleles are present in heterozygotes. High-dose refers to a requirement that the transgenic crop delivers a dose of insecticidal protein that is many times greater than the concentration required to kill heterozygotes carrying resistance alleles. The refuge part of the strategy is the requirement for farmers to grow a certain proportion of non-transgenic crop to act as a source of susceptible insects, so that any rare resistant homozygotes emerging from the transgenic crop will be highly likely to mate with the abundant susceptible homozygote from the refuge (Mendelsohn et al. 2003). The progeny of these individuals will, therefore, be heterozygotes and highly susceptible to the high dose of insecticidal protein in the transgenic crop, preventing the increase in the resistant allele frequency and outbreaks of resistant genotypes that could cause the *Bt* crop to fail (Bates et al. 2005).

Prior to the introduction of transgenic insecticidal crops, it was established that insects could become resistant to the insecticidal proteins being expressed (Tabashnick et al. 1990). Thus, the high-dose – refuge strategy is, in effect, a hypothesis that high doses of insecticidal protein and refuges of non-transgenic crops will delay the evolution of pest resistance to the protein for an acceptable period. Implicit in this hypothesis is the assumption that there is a high probability of an unacceptably rapid evolution of pest resistance should the IRM plan not be implemented. Unfortunately, this hypothesis cannot be directly tested in the field without creating the very harm one is trying to avoid. Small-scale glasshouse studies have shown that the high-dose refuge strategy can delay resistance in insect populations

(Zhao et al. 2003), leaving the current hypothesis that a particular transgenic insecticidal event or pyramid of events will delay resistance to the protein for an acceptable period. Given that resistance evolution in an insect population is driven by many uncontrollable external factors, efforts should be focused on testing hypotheses based on parameters we can measure or control. The most relevant hypotheses to test are that the plant produces protein at a high dose, the movement and mating behavior of the pest being controlled is compatible with the IRM strategy, and resistance alleles are sufficiently low. A negative or unexpected outcome from any one or all of these tests does not mean resistance cannot be sufficiently delayed, only that modifications to refuge size, configuration or proximity to the transgenic insecticidal trait fields may be required. These decisions are made with the aid of computer simulation models which help predict the relative impact of proposed IRM plans based on a given set of parameters.

15.4 Differences Between Regulatory and Research Science

Risk assessment is not scientific research and does not create scientific knowledge for its own sake (Hill and Sendashonga 2003). Instead, it organizes existing information, along with sufficient new observations, to help decision-making. It follows that while regulatory and research science both test hypotheses that are tentative solutions to problems, there are important differences between them, which if not recognized, will lead to inefficient and ineffective risk assessment and uninteresting scientific research (Raybould 2010).

Differences between regulatory and research science arise at all stages of knowledge production (Table 15.1). First, problem selection should be explicitly subjective in regulatory science because risk assessments estimate the likelihood and seriousness of harm, which is subjective. If harmful effects are not defined at the start of a risk assessment, regulatory science tends to become an effort to exhaustively characterize the effects of cultivating a transgenic crop instead of estimating the probability of harmful effects of cultivating the crop. Examples of the absence of a priori definitions of harm include the farm-scale evaluations (FSEs) of herbicide-tolerant crops in the UK (Firbank et al. 2003), many field studies that compare the abundance of non-target organisms in fields of transgenic and non-transgenic crops (e.g., Marvier et al. 2007), and the use of "omic" profiling to compare transgenic and non-transgenic crops (Ricroch et al. 2011). In each case, the research searched for differences, not potentially harmful differences. This approach is detrimental to risk assessment because differences between the transgenic and non-transgenic crops cannot be assigned a level of risk (as is the case with the meta-analysis non-target organism studies by Marvier et al. 2007), or because a subset of differences is selected after the experiment as being important (as in the FSEs), which means that resources were wasted measuring things that were irrelevant for risk assessment, that better experiments could have been designed to measure important endpoints, or both (Raybould 2007).

Table 15.1 Differences between research and regulatory science

	Research science	Regulatory science
Problem selection	• Apparently objective – Arises from objective testing of prior problems	• Subjective – Arises from definitions of harm
Hypothesis formulation	• Seeks to be interesting – Makes precise predictions – Tests fundamental theory	• Seeks to help decision-making – Predicts no harm – GMOs are not inherently harmful
Testing	• Strong corroboration from presence of phenomena in field studies • Usually requires new data	• Strong corroboration from absence of phenomena in laboratory studies • Existing data are often sufficient

Problem selection may appear objective in research science because the knowledge it produces is not ascribed obviously subjective values such as whether or not it indicates potential harm or benefit. However, problem selection will always be subjective because it is influenced by the personal interests of scientists and by organizations funding research. Apparent objectivity is not a problem for research, but is a problem in regulatory science if it induces avoidance of definitions of harm at the beginning of a risk assessment.

Owing to the different types of problem to be solved, the hypotheses tested in regulatory and research science should often be very different. Research hypotheses seek to make interesting predictions. "Interesting" science is not easy to define. However, it is often associated with precise predictions, which means that the hypothesis is exposed to falsification (Popper 1979). It is easy to make accurate, but uninteresting, predictions; for example, that in southern England, the temperature is unlikely to fall below −10°C between June and August in any given year. Many observations would corroborate such a prediction and therefore the hypothesis behind the prediction is rather boring. Much more interesting is the precise value, time and place of the minimum temperature on any given day. There are many observations that would falsify the hypothesis behind the prediction, and therefore if the hypothesis is corroborated, something interesting has been discovered.

Regulatory science seeks to help decision-making by predicting the likelihood of harmful effects. For a given decision, perhaps whether to grow a certain type of plant in one's garden, it may be sufficient to know that the temperature is unlikely to fall below −10 °C over a given period, and if so, there is no need to develop and test hypotheses about the precise minimum temperature on a given day. The same applies to risk assessment; for example, if one has tested the hypothesis that an insecticidal protein is not toxic to a valued aquatic non-target organism at concentrations in excess of ten times the maximum mean concentration of the protein produced in a transgenic crop, it is probably unnecessary to develop and test hypotheses about the precise concentration of the insecticidal protein that will appear in water bodies following cultivation of the crop. Predicting the exact number of

hybrids between a transgenic crop and a wild species, instead of the likelihood that any hybrids will form is a similar example of over-quantification of an endpoint. Clear thresholds for decisions, and simple tests of the likelihood of being above or below the threshold, are more effective for decision-making than precise predictions without an indication of which values would indicate harm.

Another problem that arises in research using transgenic crops is that the motivation for studies is that the crop is transgenic, not that the transgenic crop is a useful tool for testing an interesting hypothesis. As pointed out above, exhaustive categorization of the effects of cultivating a transgenic crop often does not help risk assessment; neither does it help research unless there is an interesting hypothesis under test. In the FSEs, the hypothesis under test was that "GMHT [genetically modified herbicide-tolerant] crops had no effect on farmland biodiversity compared with a conventional cropping system" (Squire et al. 2003). This hypothesis was highly unlikely to be true given that different herbicide management was to be applied to the conventional and transgenic crops. However, because transgenic crops were involved, the study perhaps seemed to be interesting even though there was no attempt to develop and test hypothesis from existing knowledge. Similar problems face many studies that compare transgenic and non-transgenic plants using methods that sample multiple endpoints, ranging from metabolomics to faunistic analyses at the field- or landscape-scale (Raybould 2010).

Finally, the testing of hypotheses may differ between regulatory and research science, particularly in the type of study that provides strong corroboration of a hypothesis and in the use of existing data. In regulatory science, a strong case can be made that if no potentially adverse effects are observed in controlled laboratory experiments, then field studies should not be required to demonstrate low risk from the cultivation of transgenic crops. Laboratory studies are designed to exaggerate hazards and controlled conditions mean that the effects of those hazards are more likely to be observed; this is the basis of tiered testing (Raybould 2006, 2007; Garcia-Alonso et al. 2006; Romeis et al. 2008; Raybould et al. 2011a). This does not mean that no field testing of transgenic crops is needed. A corollary of the argument for tiered testing for risk assessment is that laboratory experiments demonstrating efficacy only indicate the potential for efficacy in the field; therefore, extensive field trials are necessary to test the agronomic performance of the crop, even though laboratory tests may have shown that the crop is highly efficacious. Similarly, in ecological research laboratory testing is always likely to reveal an effect of a factor if conditions are sufficiently extreme, but this does not mean that the factor will produce that effect in the field or that the effect is ecologically important (Peters 1991). Field testing is required to demonstrate the ecological relevance of effects detected under laboratory conditions.

The important difference between regulatory science for risk assessment and efficacy trials and ecological research is that regulatory sciences usually test hypotheses that effects do not occur, whereas efficacy trials and ecological research usually test hypotheses that effects will occur. The most rigorous tests of hypotheses for the absence of effects tend to be laboratory studies, while the most rigorous tests of hypotheses for the presence of effects tend to be field studies. In both regulatory and

research science, if an effect is observed in the laboratory, its ecological importance should be evaluated in the field, and if no effect is observed in the laboratory field testing is unlikely to find an effect; therefore, while regulatory science tends to emphasize laboratory studies and research science tends to emphasize field studies, the reasoning is the same, only the hypotheses are different.

Finally, regulatory science and research science tend to differ in the use of existing data. In basic ecological research, existing data may provide good tests of new hypotheses. However, convincing corroboration of a hypothesis usually requires new experimental tests as well as re-interpretation of existing data. The data may not be in a form that provides the best test of a new hypothesis and may have been used in formulation of the hypothesis. In risk assessment, on the other hand, it is often possible and desirable to use only existing data to provide satisfactory corroboration of a risk hypothesis. In the case of a transgenic crop producing a non-pesticidal protein, for example, the risk hypothesis that the protein has no adverse effects on wildlife at concentrations in the crop can be tested using existing data on mode-of-action, amino acid sequence similarity to known toxins, and the taxonomic distribution of similar proteins (Craig et al. 2008; Raybould et al. 2010). And in the case of a transgenic crop newly developed to produce an insecticidal protein that has been extensively tested for non-target organism risk assessments for other transgenic crops, additional non-target organism studies should not be required, provided that the concentration of the insecticidal protein in the new crop is not greater than in the other crops, and provided that the species tested adequately cover the taxonomic and functional groups of non-target organisms likely to be exposed to the protein *via* cultivation of the new crop (Romeis et al. 2009).

15.5 Relevance to a Current Regulatory Problem: Combined Insect-Resistance Traits

New transgenic crops are continually being developed. Effective regulatory risk assessment and decision-making for new crops should apply experience of transgenic crops currently in commercial cultivation so that regulatory authorities are not overwhelmed reviewing studies that add little to our knowledge of the risks posed or likely benefits gained by cultivating the new transgenic crops. Transgenic crops with single insect-control traits were first commercialized over 15 years ago (Mendelsohn et al. 2003), and crops containing combinations of insecticidal traits (pyramids or stacks depending on whether the traits have overlapping or non-overlapping spectrums) are being produced by conventional breeding and are entering commercial cultivation (Halpin 2005; Gatehouse 2008). Combinations of traits may extend the range of insects controlled; for example, in maize, traits that control Lepidoptera are often combined with traits that control corn rootworm. Traits with different modes of action against the same pests may also be combined to reduce the probability of pests evolving resistance; this tactic is increasingly being used in transgenic maize and cotton resistant to Lepidoptera (Kurtz et al. 2007; Head and Dennehy 2010).

Transgenic crops containing combinations of approved traits often require additional regulatory approvals before they may be cultivated (De Schrijver et al. 2007; Taverniers et al. 2008). As the number of products with unique combinations of insect-control traits is likely to be high, an important question is whether data are required to assess the risks from cultivating a crop with two or more insecticidal proteins in addition to those used to assess the risks from cultivation of crops containing the single traits. Below we consider approaches to assessing ecological risk and developing insect-resistance management plans for crops with combinations of insect-resistant traits.

15.5.1 Ecological Risks

One way to approach the problem of assessing the ecological risks from combined insect-resistance traits is to consider the hypothesis that the ecological risk of the insecticidal traits in combination is no greater than the combined ecological risk posed by the traits separately; *i.e.*, there is no synergism between the insecticidal proteins, and the concentrations of the insecticidal proteins in the separate events are no greater than in the pyramid or stack (Raybould et al. 2011b). If these conditions hold, then if the insecticidal proteins separately have no adverse effect on non-target organisms at high concentrations relative to the concentration in the single events, the mixture of proteins is also likely to have no adverse effects on non-target organisms exposed *via* cultivation of the pyramid or stack. If the proteins separately have adverse effects on non-target organisms at concentrations likely to result from cultivation of the crop, there are methods to predict the effects of the mixture of proteins from their separate effects (Wolt 2011).

The key question for this approach is which data are needed to test the hypothesis of no synergism between the insecticidal proteins. For proteins that have no adverse effects at high concentrations relative to the crop, a mixture of proteins at the concentration in the crop is unlikely to show synergism because synergism is rarely, if ever, detected in mixtures of chemicals below their no observed adverse effect concentrations (Syberg et al. 2009); therefore, one could argue that no test of the hypothesis of no synergism should be required to assess the ecological risks from cultivating plants containing those proteins.

If additional data are required to assess the risk to non-target organisms, species that are sensitive to at least one of the proteins provide the most rigorous tests of the hypothesis of no synergism. These species are likely to be pests; however, it is their sensitivity to the proteins that is important, not whether they are non-target organisms. Several methods are available for testing the hypothesis of no synergism depending on whether the proteins have overlapping (e.g., Colby 1967; Herman et al. 2002; Fernández-Luna et al. 2010) or non-overlapping spectra (Raybould et al. 2011b), and if overlapping spectra, whether the proteins have similar or different modes-of-action (Bliss 1939; Tabashnik 1992). If no synergism is detected using sensitive species – pests or non-pests – then there is highly unlikely to be synergism

Table 15.2 Criteria for choosing testing strategies for ecological risk assessments for the cultivation of crops with combined insect-resistance traits

	Existing effects data	Number of active ingredients	Number of target groups	Combination strategy
Synergism tests	Yes	2	1	Breeding of separate events
Effects tests with mixture	No	Many	Several	Single transformation event

in non-target organisms that are insensitive to all of the proteins (Syberg et al. 2009; Raybould et al. 2011b). Such a result would provide strong corroboration of the hypothesis that two or more traits that separately pose minimal ecological risk would also pose minimal ecological risk when combined in a stack.

Finally, it should be emphasized that the ecological risk assessment does not necessarily need information on whether there is synergism among the proteins in a pyramid or stack. Tests for synergism are a means to establish whether existing data on the effects of the insecticidal proteins separately are applicable to the proteins when combined. It is perfectly possible to treat the mixture of proteins as a new active ingredient and test its effect in a series of representative surrogate organisms as is normal for single protein active ingredients (e.g., Romeis et al. 2008). This approach might be the most effective for products in which the active ingredient is a so-called binary toxin consisting of two proteins that separately have low pesticidal activity, but have high activity when combined, and when the two proteins are expressed from genes on a single DNA insert in the transgenic crop. Transgenic maize producing a toxin comprising a 14 kDa protein Cry34Ab1 and a 44 kDa protein Cry35Ab1 is an example of such a product (Moellenbeck et al. 2001; Herman et al. 2002). Table 15.2 lists some criteria that may be used to decide on testing strategies for ecological risk assessments for pyramids and stacks.

15.5.2 Insect Resistance Management for Combined Insect-Resistance Traits

In many countries, IRM plans are a compulsory part of regulatory submissions for insect-resistant transgenic crops. Such plans synthesize information about the sensitivity of the pest to the insecticidal protein. In a recent article commissioned by the Insect Resistance Action Committee, MacIntosh (2010) outlines the types of data needed to develop an effective IRM plan adapted to local environments. The author states that in regard to IRM plans and regulation, “The goal should be to enable growers to have access to the technology while providing stewardship that will provide an acceptable level of protection against resistance”. The key areas where data are needed to fulfill that goal are outlined in the article, and include understanding primary pest biology and ecology, potential trait use patterns, local cropping and patterns systems, dose (level of target pest control) and number of insecticidal

proteins expressed by the plant, and the potential for cross resistance between insecticidal proteins.

Of particular importance to an IRM plan is the dose of insecticidal protein delivered by the product. When combining insect-control traits, it is important to predict whether stacking or pyramiding single events will have an impact on the dose of each individual insecticidal protein expressed by the product. In some regions, data may not exist for the single traits and should be generated; however, in regions where single traits have previously been commercialized, the existing data can be used to inform regulatory risk assessment and decision-making for combined insect-resistance traits (stacks or pyramids) rather than generating new data on the dose of protein expressed by single traits.

If the dose of insecticidal protein delivered by each commercial single trait against key pests has previously been determined, it should not be necessary to repeat additional dose studies for the combined insect-resistance traits. A quantitative assay comparing protein concentrations in products with the single trait events to those expressing the combined insect-resistance traits should be sufficient. If expression of the proteins in the stacked product is comparable to expression of each protein in the single-trait events, insect pests will receive the same dose of insecticidal protein given that no synergism or antagonism was observed in the studies described in the section above. In the case of IRM plans for stacked products with non-overlapping spectra, the IRM plans developed for the single traits can apply directly to the stacked product. For pyramided products with overlapping spectra, the dose of the single trait events can be used to determine what IRM plan would be most appropriate.

Though the existing data on single traits can be used to inform regulatory risk assessment and decision-making for combined insect-resistance traits, ensuring that the combined stacks or pyramids are performing as expected is also important. The results of the quantitative protein assays are often supplemented with standard field efficacy trials comparing the performance of the stack or pyramid to the known performance of each single trait component.

15.6 Conclusion

When regulatory science is confused with research, many irrelevant data may be produced, which confuse and delay decision-making, and increase the costs of regulation to the developer and regulator, ultimately harming innovation because business risks are too high. High costs are particularly problematic for public sector institutions and small companies that cannot afford the regulatory costs even if they wished to run the business risks (Kalaitzandonakes et al. 2007). On the other hand, if research is confused with regulatory science, uninteresting hypotheses are tested, which slow the development of knowledge, again harming innovation. Good regulatory and research science should be directed by the formulation and testing of suitable hypotheses, but it is important that the objectives of research to test scientifically interesting hypotheses are not confused with the objectives of regulatory

science to test hypotheses that help decision-making. In some cases, particularly very early in the development of new technology, regulatory science and research may be indistinguishable; however, it is important for the effective development of new technology that regulatory data requirements are not laid down to answer research questions.

We are now beginning to see clarification of the concepts that allow identification of data essential for risk assessment of transgenic crops ("need to know information") and data that may appear useful for a risk assessment, but at best are irrelevant, and at worst create delay and confusion in decision-making ("nice to know information"). As new technologies such as synthetic biology and nanotechnology are applied to agriculture, it is essential that regulation of resulting products learns from the experience with transgenic crops. Science can help to assess the risks from new classes of product, but thinking that objective scientific knowledge is all that is needed to make good decisions is mistaken; subjective elements are needed to decide what to regard as harmful effects and to set decision-making criteria. Often difficult decisions require clear thinking about the nature of the problem: what do we want and how should we decide whether we are likely to get it? If we are unsure what we want, more information is likely to confuse us rather than clarify the choices. We have found this to be the case with transgenic crops, and we must not forget it when deciding whether or how new agricultural technologies should be regulated.

References

Bates SL, Zhao J-Z, Roush RT, Shelton AM (2005) Insect resistance management in GM crops: past, present and future. Nat Biotechnol 23:57–62

Bliss CI (1939) The toxicity of poisons applied jointly. Ann Appl Biol 26:585–615

Brookes G, Barfoot P (2008) Global impact of biotech crops: socio-economic and environmental effects 1996–2006. AgBioForum 11:21–38

Brookes G, Barfoot P (2010) Global impact of biotech crops: environmental effects, 1996–2008. AgBioForum 13:76–94

Caley P, Lonsdale WM, Pheloung PC (2006) Quantifying uncertainty in predictions of invasiveness, with emphasis on weed risk assessment. Biol Invasions 8:1595–1604

Chapman PM, Fairbrother A, Brown D (1998) A critical evaluation of safety (uncertainty) factors for ecological risk assessment. Environ Toxicol Chem 17:99–108

Chataway J, Tait J, Wield D (2006) The governance of agro- and pharmaceutical biotechnology innovation: public policy and industrial strategy. Technol Anal Strateg Manag 18:169–185

Colby SR (1967) Calculating synergistic and antagonistic response of herbicide combinations. Weeds 15:20–22

Craig W, Tepfer M, Degrassi G, Ripandelli D (2008) An overview of general features of risk assessments of genetically modified crops. Euphytica 164:853–880

De Schrijver A, Devos Y, Van den Bulcke M, Cadot P, De Loose M, Reheul D, Sneyers M (2007) Risk assessment of GM stacked events obtained from crosses between GM events. Trends Food Sci Technol 18:101–109

Federoff NV, Battisi DS, Beachy RN, Cooper PJM, Fischhoff DA, Hodges CN, Knauf VC, Lobell D, Mazur BJ, Molden D, Reynolds MP, Roland PC, Rosegrant MW, Sanchez PA, Vonshak A, Zhu J-K (2010) Radically rethinking agriculture for the 21st century. Science 327:833–834

Fernández-Luna M, Tabashnik BE, Lanz-Mendoza H, Bravo A, Soberón M, Miranda-Ríos J (2010) Single concentration tests show synergism among *Bacillus thuringiensis* subsp. *israelensis* toxins against the malaria mosquito *Anopheles albinamus*. J Invert Pathol 104:231–233

Firbank LG, Heard MS, Woiwod IP, Hawes C, Haughton AJ, Champion GT, Scott RJ, Hill MO, Dewar AM, Squire GR, May MJ, Brooks DR, Bohan DA, Daniels RE, Osborne JL, Roy DB, Black HIJ, Rothery P, Perry JN (2003) An introduction to the farm-scale evaluations of genetically modified herbicide-tolerant crops. J Appl Ecol 40:2–16

Fuchs M, Gonsalves G (2007) Safety of transgenic virus-resistant plants two decades after their introduction: lessons from realistic field risk assessment studies. Annu Rev Phytopathol 45:173–202

Garcia-Alonso M, Jacobs E, Raybould A, Nickson TE, Sowig P, Willekens H, Van der Kouwe P, Layton R, Amijee F, Fuentes A, Tencalla F (2006) Assessing the risk of genetically modified plants to non-target organisms. Environ Biosafety Res 5:57–65

Gatehouse JA (2008) Biotechnological prospects for engineering insect-resistant plants. Plant Physiol 146:881–887

Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C (2010) Food security: the challenge of feeding 9 billion people. Science 327:812–818

Halpin C (2005) Gene stacking in transgenic plants. Plant Biotechnol J 3:141–155

Head G, Dennehy T (2010) Insect resistance management for transgenic Bt cotton. Biotechnol Agric For 65:113–125

Herman RA, Scherer PN, Young DL, Mihaliak CA, Meade T, Woodsworth AT, Stockhoff BA, Narva KE (2002) Binary insecticidal crystal protein from *Bacillus thuringiensis*, strain PS149B1: effects of individual protein components and mixtures in laboratory bioassays. J Econ Entomol 95:635–639

Hill R, Sendashonga C (2003) General principles for risk assessment of living modified organisms: lessons from chemical risk assessment. Environ Biosafety Res 2:81–88

Johnson KL, Raybould AF, Hudson MD, Poppy GM (2007) How does scientific risk assessment of GM crops fit within the wider risk analysis? Trends Plant Sci 12:1–5

Kalaitzandonakes N, Alston J, Bradford K (2007) Compliance costs for regulatory approval of new biotech crops. Nat Biotechnol 25:509–511

Kurtz RW, McCaffery A, O'Reilly D (2007) Insect resistance management for Syngenta's VipCot™ transgenic cotton. J Invert Pathol 95:227–230

MacIntosh SC (2010) Managing the risk of insect resistance to transgenic insect control traits: practical approaches in local environments. Pest Manag Sci 65:100–106

Marvier M, McCreedy C, Regetz J, Kareiva P (2007) A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. Science 316:1475–1477

McGaughey WH, Whalon ME (1992) Managing insect resistance to *Bacillus thuringiensis* toxins. Science 258:1451–1455

McGaughey WH, Gould F, Gelernter W (1998) *Bt* resistance management. Nat Biotechnol 16:144–146

Mendelsohn M, Kough J, Vaitusis Z, Matthews K (2003) Are *Bt* crops safe? Nat Biotechnol 21:1003–1009

Mittra J, Tait J, Wield D (2011) From maturity to value-added innovation: lessons from the pharmaceutical and agro-biotechnology industries. Trends Biotechnol 29:105–109

Moellenbeck DJ, Peters ML, Bing JW, Rouse JR, Higgins LS, Sims L, Nevshemal T, Marshall L, Ellis RT, Bystrak PG, Lang BA, Stewart JL, Kouba K, Sondag V, Gustafson V, Nour K, Xu D, Swenson J, Zhang J, Czapla T, Schwab G, Jayne S, Stockhoff BA, Narva K, Schnepf HE, Stelman SJ, Poutre C, Koziel M, Duck N (2001) Insecticidal proteins from *Bacillus thuringiensis* protect corn from corn rootworms. Nat Biotechnol 19:668–672

Peters RH (1991) A critique for ecology. Cambridge University Press, Cambridge

Popper KR (1959) The logic of scientific discovery. Hutchinson, London

Popper KR (1979) Objective knowledge: an evolutionary approach. Oxford University Press, Oxford

Qaim M (2009) The economics of genetically modified crops. Annu Rev Resour Econ 1:665–693
Raybould A (2006) Problem formulation and hypothesis testing for environmental risk assessments of genetically modified crops. Environ Biosafety Res 5:119–125
Raybould A (2007) Ecological versus ecotoxicological methods for assessing the environmental risks of transgenic crops. Plant Sci 173:589–602
Raybould A, Stacey D, Vlachos D, Graser G, Li X, Joseph R (2007) Non-target organism risk assessment of MIR604 maize expressing mCry3A for control of corn rootworm. J Appl Entomol 131:391–399
Raybould A (2010) Reducing uncertainty in regulatory decision-making for transgenic crops: more ecological research or clearer environmental risk assessment? GM Crops 1:25–31
Raybould A (2011) The bucket and the searchlight: formulating and testing risk hypotheses about the weediness and invasiveness potential of transgenic crops. Environ Biosafety Res 9:123–133
Raybould AF, Cooper JI (2005) Tiered tests to assess the environmental risk of fitness changes in hybrids between transgenic crops and wild relatives: the example of virus resistant *Brassica napus*. Environ Biosafety Res 4:127–140
Raybould A, Quemada H (2010) *Bt* crops and food security in developing countries: realised benefits, sustainable use and lowering barriers to adoption. Food Sec 2:247–259
Raybould A, Vlachos D (2011) Non-target organism effects tests on Vip3A and their application to the ecological risk assessment for cultivation of MIR162 maize. Transgenic Res 20:599–611
Raybould A, Tuttle A, Shore ST (2010) Environmental risk assessments for transgenic crops producing output trait enzymes. Transgenic Res 19:595–609
Raybould A, Caron-Lormier G, Bohan DA (2011a) The derivation and interpretation of hazard quotients to assess the ecological risks from the cultivation of insect-resistant transgenic crops. J Agric Food Chem 59:5877–5885
Raybould A, Graser G, Hill K, Ward K (2011b) Ecological risk assessments for transgenic crops with combined insect-resistance traits: the example of Bt11 x MIR604 maize. J Appl Entomol 136:27–37
Ricroch AE, Bergé JB, Kuntz M (2011) Evaluation of genetically engineer crops using transcriptomic, proteomic and metabolomic profiling techniques. Plant Physiol 155:1752–1761
Romeis J, Bartsch D, Bigler F, Candolfi MP, Gielkens MMC, Hartley SE, Hellmich RL, Huesing JE, Jepson PC, Layton R, Quemada H, Raybould A, Rose RI, Schiemann J, Sears MK, Shelton AM, Sweet J, Vaituzis Z, Wolt JD (2008) Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. Nat Biotechnol 26:203–208
Romeis J, Lawo NC, Raybould A (2009) Making effective use of existing data for case-by-case risk assessments of genetically engineered crops. J Appl Entomol 133:571–583
Sarewitz D (2004) How science makes environmental controversies worse. Environ Sci Policy 7:385–403
Squire GR, Brooks DR, Bohan DA, Champion GT, Daniels RE, Haughton AJ, Hawes C, Heard MS, Hill MO, May MJ, Osborne JL, Perry JN, Roy D, Woiwod IP, Firbank LG (2003) On the rationale and interpretation of the farm-scale evaluations of genetically modified herbicide-tolerant crops. Phil Trans R Soc Lond Ser B 358:1779–1799
Syberg K, Jensen TS, Cedergreen N, Rank J (2009) On the use of mixture toxicity assessment in REACH and the Water Framework Directive: a review. Hum Ecol Risk Assess 15:1257–1272
Tabashnik B (1992) Evaluation of synergism among *Bacillus thuringiensis* toxins. Appl Environ Microbiol 58:3343–3346
Tabashnik BE, Cushing NL, Finson N, Johnson MW (1990) Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J Econ Entomol 83:1671–1676
Taverniers I, Papazova N, Bertheau Y, De Loose M, Holst-Jensen A (2008) Gene stacking in transgenic plants: towards compliance between definitions, terminology, and detection in the EU regulatory framework. Environ Biosafety Res 7:197–218
Touart LW, Maciorowski AF (1997) Information needs for pesticide registration in the United States. Ecol Appl 7:1086–1093

Wolt JD (2011) A mixture toxicity approach for environmental risk assessment of multiple insect resistance genes. Environ Toxicol Chem 30:763–772

Wolt JD, Keese P, Raybould A, Fitzpatrick JW, Burachik M, Gray A, Olin SS, Schiemann J, Sears M, Wu F (2010) Problem formulation in the environmental risk assessment of genetically modified plants. Transgenic Res 19:425–436

Zhao JZ, Coa J, Li Y, Collins HL, Roush RT, Earle ED, Shelton AM (2003) Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat Biotechnol 21:1493–1497

Chapter 16
The Globalization of Agricultural Biotechnology: Implications for Regulatory Compliance, Stewardship and Stakeholder Engagement

Jeffrey D. Rowe, Firoz Amijee, Steven D. Brody, Greg G. Wandrey, and Courtney Chabot Dreyer

Abstract In 1993, a Swedish researcher determined the contents of a typical Swedish breakfast traveled a distance equal to the circumference of the Earth before reaching the breakfast table.[1] Twelve years later, a researcher in Iowa found that the ingredients of a carton of strawberry yogurt collectively traveled 2,211 miles, or 3,558 km, just to reach the processing plant.[2] While the local-food movement has gained traction in some countries, the reality is that we (humanity) have a globalized food system.

Countries throughout the world import millions of metric tons of row crops and cereals such as corn, wheat, rice, soybeans and the by-products of those crops to sustain their people and economies. The US and Canada are major exporters of those crops and many other agricultural commodities and by-products. Therefore, every food producer, trader, processor, manufacturer and transporter within the US and Canadian value chains are impacted by the regulatory and political systems of the major agricultural export markets. It is not practical to think any domestic food system can act in isolation, ignoring the policies, regulations and consumer-demands in other markets.

The rapid introduction and mass-adoption of crops developed through biotechnology since 1996 have exposed the intricate relationship amongst agricultural regulatory bodies and their policies around the world. This chapter outlines the challenges of the globalization of agricultural biotechnology; discusses stewardship practices that standardize the introduction, cultivation and discontinuation of biotech

[1] Sarah DeWeerdt, "Is Local Food Better?" World Watch Magazine, May/June 2012, Vol. 22, No. 3. Available at: http://www.worldwatch.org/node/6064

[2] Id.

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products; and acknowledges the need to increase stakeholder engagement to provide more detailed and timely information about biotechnology, while also respecting and acknowledging the differences in public acceptance of biotech crops globally .

Keywords Biotechnology • Exports • Globalization • Imports • Insect resistance management • Plenish™ • Regulatory compliance • Stewardship • Trade • Stakeholder engagement

16.1 The Globalization of Agricultural Biotechnology

Unlike politics, all agricultural biotechnology is NOT local. Companies or institutions in the agricultural biotechnology industry who conduct research and development with biotech (transgenic or genetically modified) plants, with the objective of commercializing biotech seed products derived from those plants, have an obligation to understand and abide by the applicable rules and regulations of not just the countries in which they operate, but also the countries where their product may end up.

Historically, research and development functions have been primarily based in the United States (US) and Canada and are subject to the regulations governing the planting and testing of regulated materials in those countries. As described elsewhere in this book, product performance and safety evaluations are increasingly extending to international markets. Furthermore, after regulatory approval is obtained in domestic markets, the grains and oilseeds from these biotech crops will find their way into the worldwide food chain, usually as processed food products (e.g., soybean oil, corn starch, high fructose corn syrup) or as meat, milk and eggs from animals that are fed biotech crops. The markets for the commodity grains and processed foods are global. Consequently, the developer of a biotech product must have knowledge of local regulations, and must know in what other countries it will be grown and imported to, as well as how the biotech crop will be harvested, transported, processed and utilized and what regulations govern these activities in each country.

Above and beyond those legal requirements, responsible developers of biotech crops also implement stewardship practices based on industry agreed best practices and individual company policies to ensure that biotech research, development and production activities avoid the possibility of harm to the environment and to human or animal health, and disruptions to interstate commerce and international trade, while benefiting customers and global stakeholders. Stakeholders along the food value chain, up to and including the consumer, have a vested interest in regulatory compliance and good stewardship and often play a part in influencing the development of regulations and stewardship planning, implementation and compliance.

Stakeholder engagement through the entire process of research, development, commercialization and product discontinuation is not only a good business practice,

but it is critical to the long term success of biotech products in the marketplace. Everyone along the value chain, including consumers, must have confidence in the safety of biotech products that will become, directly or indirectly, components of our diet. While regulatory approvals are the basis for assuring food, animal feed and environmental safety, working with stakeholders to explain those approvals and the interplay between domestic and international requirements, addressing specific questions and providing information about the technology is also important.

16.1.1 Global Trade in Commodity Grains and Oilseeds

The days when US and Canadian farmers grew crops largely to feed animals on their own farms or to supply local food and feed processors are long gone. Today's agricultural crops are globally traded commodities, and increasing proportions of the annual crops harvested in the US and Canada are exported to satisfy the world's growing demand for food, especially for meat production from animals raised on grains and grain by-products, such as corn gluten, distillers dried grains and soybean meal.

Each year, a typical corn grower in the US or Canada will carefully select the right combination of hybrids to grow, with the appropriate combination of characteristics to maximize yield, which may include biotech traits for particular management practices (e.g., tolerance to a herbicide to facilitate no-till cultivation) and to control pests that are prevalent in the area (e.g., European corn borer and corn rootworm). At the end of the growing season, the grain from the different hybrids is harvested and aggregated. The farmer may sell some of the crop directly to a local elevator or use on-farm storage anticipating higher prices in the coming months. When the grain eventually goes to the elevator, it is graded, co-mingled with the grain from neighboring farms and then sold for domestic use or for export. Grain destined for export will be transported by truck or rail from the elevator to a grain terminal near the Mississippi River, before loading onto barges for transport to a port for further storage and finally loaded into ships destined for a grain importing country. At every step, the grain is co-mingled making it virtually impossible to trace a portion of the shipment back to the original farm where it was grown or to identify a particular hybrid the farmer planted. This is the commodity grain channel that has been in existence in the US since the early 1900s (Figs. 16.1 and 16.2, 16.3 and 16.4).

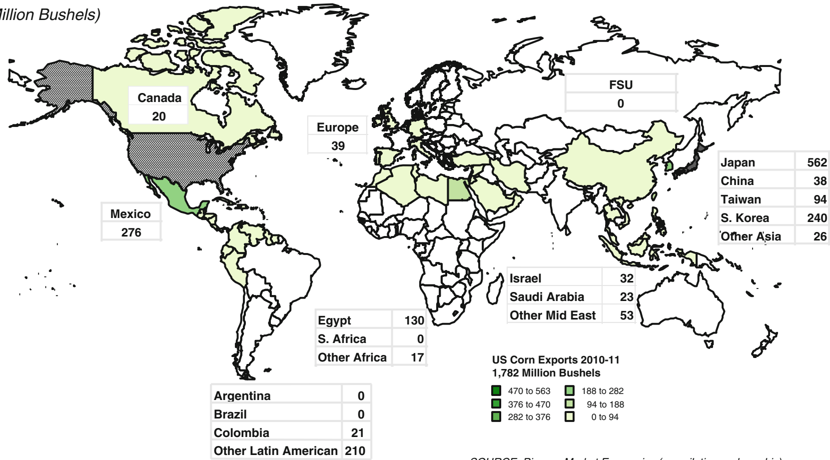
In 2008–2009, the United States Department of Agriculture (USDA) Foreign Agricultural Service (FAS) reported that 41 countries each imported more than 10,000 metric tons of US corn. Approximately 15% (45 million metric tons) of the 2008–2009 US corn harvest of 307 million metric tons was exported.[3] Most of the exported crop was destined to be used as animal feed.

[3] National Corn Growers Association World of Corn 2009 Statistics Book (http://www.ncga.com/files/pdf/WOC2009MetricStatBook.pdf) (Table 16.1).

U.S. Corn Exports in 2010/11

September-August

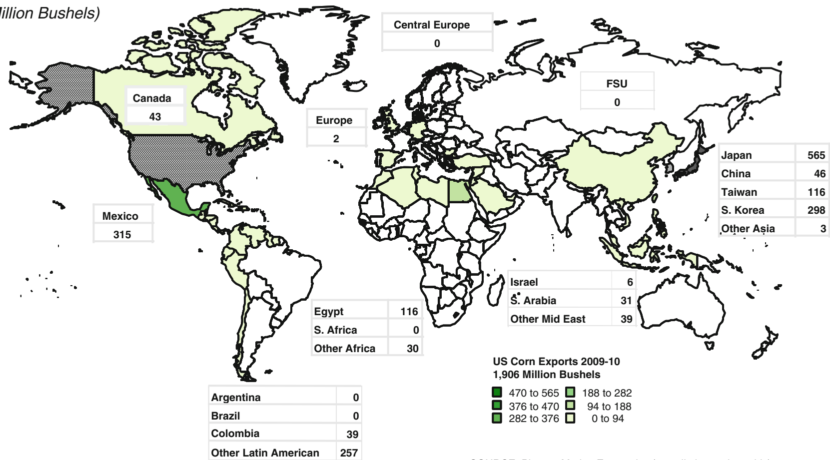
(Million Bushels)



U.S. Corn Exports in 2009/10

September-August

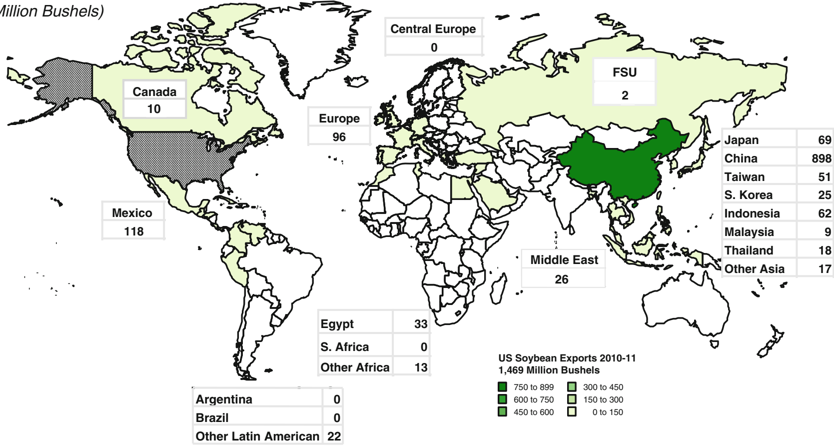
(Million Bushels)



Figs. 16.1 and 16.2 Maps showing the major destinations of US corn exports in 2010–2011 and 2009–2010

U.S. Soybean Exports in 2010/11

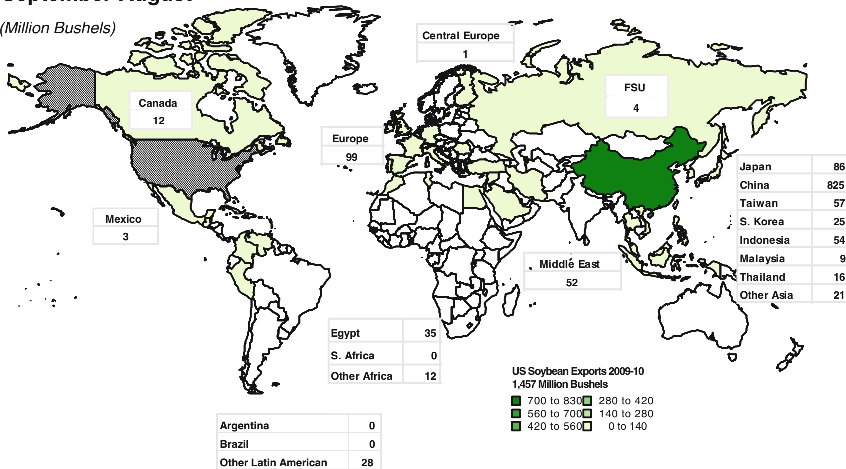
September-August



SOURCE: Pioneer Market Economics (compilation and graphic)
USDA FAS Export Sales Reports (data)

U.S. Soybean Exports in 2009/10

September-August



SOURCE: Pioneer Market Economics (compilation and graphic)
USDA FAS Export Sales Reports (data)

Figs. 16.3 and 16.4 Maps showing the major destinations of US soybean exports in 2010–2011 and 2009–2010

Soybeans are exported as whole grain for processing, or as processed products—oil, which is largely used in food processing, or the residual meal, which is used as a source of protein for animal feed.

Some countries such as Mexico, China, Japan and Korea are significant importers of US corn and soybeans (Table 16.1). This reflects their domestic demand for animal feed that cannot be met by local production. Others, such as the European Union (EU), import little or no corn, but import large amounts of soybean meal as a source of protein to supplement locally produced feed. Many export markets are extremely price sensitive and the US faces strong competition from grains and oilseeds produced in South America. Ready access to biotechnology that can increase the efficiency of production, such as herbicide tolerant crops and crops protected from insect pests, coupled with a highly developed infrastructure for the bulk transport, handling and storage of grain are key factors in the competitiveness of US and Canadian growers in international grain markets.

16.2 Global Regulatory Compliance

16.2.1 The Need for Synchronized Import Approvals

Since the advent of biotechnology, an increasing number of importing countries have developed regulations requiring the pre-import approval of raw agricultural commodities derived from biotech crops. The need for such approvals can pose significant impediments to the free flow of agricultural commodities in global commerce, unless (1) there are common requirements to gain approvals and the timing of approvals are synchronized, or (2) the importing country recognizes the approvals obtained in the country of origin and the importing country has a threshold for small amounts of biotech grain to be present, inadvertently, that do not yet have all necessary import approvals.

Although some countries have thresholds for the unavoidable presence of biotech materials in feeds for biotech products that have been approved in the country of production, or are proposing such measures, similar arrangements to allow trace amounts of unapproved biotech materials in food products are largely absent at the present. Complying with the diverse patchwork of international regulations governing the approval for import of biotechnology products for both food and feed use results in asynchronous approvals, and is one of the biggest challenges faced by the developers of biotech crops as they strive to achieve timely commercial deployment.

In practice, it is not feasible to commercialize a biotech crop variety containing a new biotech trait based solely on domestic (i.e., US and Canadian) approvals. A company must obtain the necessary international approvals to allow the import of

Table 16.1 Top ten importers of US corn, soybeans, soybean oil and soybean meal

US Corn and soybean destinations by rank 2010–2011

Corn		Soybeans		Soybean meal		Soybean oil	
Country	% total exports	Country	% total exports	Country	% total exports	Country	% total exports
Japan	31.55	China	61.12	Mexico	15.31	China	26.20
Mexico	15.51	Mexico	8.04	Canada	12.99	Morocco	21.85
S. Korea	13.49	Japan	4.72	Philippines	11.92	Mexico	8.80
Egypt	7.30	Indonesia	4.20	Venezuela	7.71	Algeria	8.45
Taiwan	5.29	Taiwan	3.49	Morocco	6.60	Dom. Rep.	5.71
Syria	2.29	Netherlands	2.43	Japan	4.72	Colombia	4.24
China	2.16	Egypt	2.24	Dom. Rep.	4.53	Venezuela	3.62
Venezuela	1.97	Spain	2.14	Guatemala	3.36	Peru	3.39
Israel	1.77	South Korea	1.73	Ecuador	2.52	Guatemala	2.91
Dom. Rep.	1.51	Thailand	1.21	South Korea	2.30	Canada	2.74

Source: USDA Foreign Agricultural Service

grain from that biotech crop into key US and Canadian export markets. As a matter of stewardship, many technology developers have committed not to launch commercialization of biotech crops without key international import approvals[4] sometimes depriving domestic growers of timely access to new technology. Several other countries (e.g., China) are developing biotech crops for domestic production, which in the future, are likely to find their way into commodity streams destined for the US and Canada. The US system for evaluating imported biotech commodities developed outside of the US remains untested, and at this time it is not clear how the USDA will approve biotech grains for import, address the presence of unapproved biotech material in imported commodities or how it will establish thresholds for such material.

16.2.2 The Global Patchwork of Regulatory Systems

Even if biotech crops are intended to be grown primarily for domestic use, any unintended presence of that grain into the export supply could result in negative consequences for international trade. For this reason, developers seek import approvals of key countries before the unrestricted commercialization of biotech crops occurs. This presents a major challenge in view of the diversity of regulations governing agricultural biotechnology products administered by multiple agencies within each country and the differing definitions of what is regulated. Moreover, the mechanisms for conducting bio-safety assessments, the data requirements and timing of approvals also vary from country to country. Against this background of complexity and uncertainty, the developer of biotech crops must anticipate when approvals will be received so that the timing of seed production and sales in the US or Canada can be synchronized with approvals in major import markets. Depending on the crop, it takes several seasons to produce enough seed for a commercial launch, and great care has to be taken to ensure that seed grown for a regulated biotech crop does not get mixed, inadvertently, with commodity grain during the cycles of seed multiplication.

Biotechnology has been regulated in an extremely proactive manner. This reflects the intense public debate about the utility and perceived risks of the technology, especially as it impacts the food supply and the environment—a debate which has arguably been intensified in the public's mind by the leadership role of multinational companies in the development of biotech products, rather than the public sector. In fact, the stringency of the regulations in certain countries and the associated costs has meant there is little room for the public sector to resource the development of biotech crops and to comply with the stringent global regulatory requirements.

[4] http://www2.dupont.com/Biotechnology/en_US/positions/position_statements.htm

The global debate on the safety of agricultural biotechnology could have provided an opportunity for the parallel development of harmonized regulatory systems with opportunities for extensive reciprocal recognition of scientific assessments of bio-safety and determinations of safety. Instead, despite the best efforts of international bodies such as the Organization for Economic Cooperation and Development (OECD), each jurisdiction set about crafting their own regulations and supplementary requirements, with little recognition for the unprecedented record of safety and advances in other countries.

A significant point of disparity became the discussion as to whether regulations should be based on "product or process." The US and Canada, in particular, argued that it was the nature of the product that was critical in determining an appropriate regulatory system, and that existing regulations governing food and feed in commerce could be amended to include oversight of new and novel products, developed using biotechnology and the application of recombinant DNA (deoxyribonucleic acid). Other countries, led by the EU, believed that the process by which the product was derived was the critical factor in developing regulatory oversight, irrespective of whether there was a material change in the property of the food or feed. Since biotechnology was new and unfamiliar, they proposed that a new regulatory system be developed to evaluate the safety of biotech products. Even today, the EU continues to consider extending their regulatory remit to take new developments in biotechnology into account. Countries that focused on the nature of the product and adapting existing legislation (e.g., US and Canada) were able to have functioning regulatory systems in place well before many of the countries that chose to try to address all perceived risks in new regulations by assessing the process by which biotech products were developed (e.g., EU).

Discussions continue over the appropriate level of regulatory oversight for agricultural biotechnology that is now widely adopted by more than 16 million farmers in 29 countries on 160 million hectares.[5] In 2000, the EU announced that henceforth they would apply the Precautionary Principle (Principle) to regulatory oversight for biotech products. The Principle "…covers cases where scientific evidence is insufficient, inconclusive or uncertain and preliminary scientific evaluation indicates that there are reasonable grounds for concern that the potentially dangerous effects on the environment, human, animal or plant health may be inconsistent with the high level of protection chosen by the EU."[6] The practical application of the Principle has become a controversial issue in trade relations between the US and the EU. Critics of the Principle (mainly the US) are suspicious that any uncertainty can be used to invoke the Principle and that the standard of sufficient scientific evidence to address a risk is not defined and can be set so high as to be impractical to meet. This difference of opinion has caused disruptions in trade between the US and the EU.

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[5] James, Clive. International Service for the Acquisition of Agri-Biotech Applications, "Global Status of Commercialized Biotech/GM Crops: 2011."

[6] http://europa.eu/rapid/pressReleasesAction.do?reference=IP/00/96&format=HTML&aged=0&language=EN&guiLanguage=en

16.2.3 The Review Process

Apart from the philosophical differences in approach to biotech regulations, countries have developed fundamentally different organizations and mechanisms for conducting reviews. Most countries separate environmental safety assessments from assessments of food and feed safety and different agencies are typically responsible for these reviews. In 1986, the US created the Coordinated Framework for Regulation of Biotechnology allocating different areas of responsibility between the key regulatory agencies—USDA, Food & Drug Administration (FDA) and the Environmental Protection Agency (EPA).[7] The Coordinated Framework was created to make best use of available experience and expertise, share opportunities for expanded oversight and limit potential duplication. Most other countries have also elected to split accountabilities between agencies. Ministries of Health are usually made accountable for regulating foods derived from biotech crops; Ministries of Agriculture typically deal with feed safety where feed use is specifically regulated; and Ministries of Agriculture, or more often the Ministries of the Environment, are accountable for the oversight of environmental safety. A few countries (e.g., Argentina) also require a market assessment to evaluate the economic impact of approving new biotech crops.

It is imperative for technology developers to understand how each entity functions to obtain all requisite approvals from each organization in the appropriate countries to enable functional deregulation and global commercialization of biotech crops (Table 16.2).

16.2.4 Different Approaches to Reviewing Biotech Product Safety Data

At the heart of any biotech regulatory system is the technical review of information provided by the applicant in support of the product's safety upon release into the environment and as a food and/or animal feed. The US and Canada typically rely on career civil service scientists to act as reviewers, but many other countries have created panels or advisory committees of academic experts with technical expertise in different areas. These expert bodies are typically charged with making recommendations to a secretariat or an administration within a ministry or authorizing department. Opinions will vary as to the merits of either system. Regulatory systems that make use of full-time staff reviewers may be more consistent in how they review and analyze the scientific information supporting product safety. On the other hand, external panels can be better informed on the latest science, but may not always appreciate the restrictions on subject matter imposed by the scope of a regulation.

The steady increase in the number of new products seeking regulatory approval has placed strains on all review bodies. Regulatory agencies such as USDA-APHIS-BRS

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[7] See: http://usbiotechreg.nbii.gov/CoordinatedFrameworkForRegulationOfBiotechnology1986.pdf

Table 16.2 Global approvals for glyphosate tolerant soybean event MON-04032-6 (GTS 40-3-2)

Country	Environment	Food	Feed
Argentina	1996	1996	1996
Australia/NZ		2000	
Brazil	1998	1998	1998
Canada	1995	1996	1995
China		2004	
Columbia		2005	
Czech Republic		2001	2001
European Union		1996	
Japan	1996	1996	1996
Korea		2004	2004
Mexico	1998	1998	1998
Paraguay	2004	2004	
Philippines		2003	2003
Russia		1999	
South Africa	2001	2001	2001
Switzerland		1996	1996
Taiwan		2002	
United States	1994	1994	
Uruguay	1997	1997	1997

Information compiled from: http://bch.cbd.int/database/record-v4.shtml?documentid=14796

(Animal and Plant Health Inspection Service, Biotechnology Regulatory Service) continually struggle to fund, hire, train and retain qualified reviewers, while academics serving as experts are increasingly concerned by the numbers of review meetings and the sheer volume of materials they are expected to evaluate while managing their academic careers. As a consequence, most regulatory organizations have trouble finding sufficient resources necessary to ensure a timely turnaround of applications.

16.2.5 The Importance of Understanding the Local Process and Issues

A developer of biotech products must understand how the regulatory organizations in each country are structured; the process for review and approval; and the data that are likely to be required in order to answer the questions posed by the reviewers. As discussed, the organization and process is unique for each country (See Appendix). For example, Japan and the EU both make use of panels of technical experts, but their review processes are completely different (See Appendix).

Many technology developers are multinational companies that are also interested in commercializing biotech seed products in markets outside of the US and Canada, such as Argentina, Brazil, China, Europe, India, Mexico and South Africa. Separate environmental approvals may be required to cultivate a biotech crop than those necessary to import commodity grain. This is reflected in additional environmental questions that arise when large numbers of fertile plants with biotech traits will be introduced into an agro-ecosystem with a focus on the potential for outcrossing and persistence of the biotech traits in cultivated or wild species (e.g., canola outcrossing to *Brassica* species in Europe, soybean outcrossing to *Glycine spp*. in S.E. Asia and corn outcrossing to *Zea* species in Mexico). To address these questions, developers may be required to conduct contained or confined tests of biotech crops in those countries from which they seek cultivation approval. The safety assessments lead to endpoints, which address any of these potential questions.

Generally the approvals obtained for import of food and feed materials can be also used to support commercial cultivation of a biotech crop, since the ways in which humans and animals metabolize plant-based foods generally does not vary with geography. However, risk assessments should take into account marked differences in dietary exposure in different regions and the associated risk of allergies developed to commonly consumed foods. For example, peanuts are a common allergen in the US, whereas buckwheat allergy is common in Japan, due to the consumption of soya noodles containing buckwheat flour. Regulatory systems generally account for these differences.

16.2.6 Prospects for Harmonization of Data Requirements

The information that must be supplied to satisfy the requirements for biotech product safety evaluations in each jurisdiction, are often not well defined and continually evolve. A number of scientific panels have been convened to consider harmonization of data requirements under the auspices of organizations such as OECD, the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (UNFAO). Resulting guidelines such as Codex Alimentarius (2003) Principles for Risk Analysis and Guidelines for Safety Assessment of Foods Derived from Modern Biotechnology have been valuable in defining common principles and promoting consistency in the information required to conduct safety evaluations.[8] Some agencies also publish and regularly update guidelines to assist applicants such as the European Food Safety Authority (EFSA) Guidance Document of the Scientific Panel on Genetically Modified Organisms for the Risk Assessment of Genetically Modified Plants and Derived Food and Feed.[9] The technology is, however, very dynamic and constantly evolves and incorporates new techniques that can sometimes

[8] ftp://ftp.fao.org/codex/Publications/Booklets/Biotech/Biotech_2003e.pdf

[9] http://www.efsa.europa.eu/en/scdocs/scdoc/99.htm

raise new questions about the safety of the product, which sometimes spurs new data requirements.

Different jurisdictions may interpret international guidelines differently or have additional scientific considerations they believe important to address in a local context. The composition of experts on review committees and the research interests of the current members can also influence data requirements. Ultimately, the list of studies that must be conducted to generate the data necessary to satisfy the requirements of all the key regulatory authorities globally is dynamic. Developers must carefully review the publicly available information on current applications to stay abreast of recent developments and should take advantage of any opportunities to consult directly with regulators to anticipate data requirements. Regulators' attitudes toward consultations with developer-applicants vary considerably. Some agencies encourage consultations to improve quality and content of submissions or to resolve questions during the review process. Others actively discourage contact between the agency technical staff and applicants, preferring to keep administrative secretariats positioned between the applicant and reviewer.

16.2.7 Data Requirements and Costs

The list of bio-safety studies in Table 16.3 is intended to convey the scope of activities that must be undertaken and is not a definitive or exhaustive list. For every product, the list of studies should reflect the nature of the product and the environments in which it will be grown. For example, outcrossing to wild corn species is a risk that must be evaluated for a new corn product to be grown in Mexico, but a study on outcrossing is not necessary if the crop will be grown in the continental US, where there are no wild corn relatives. A crop engineered with a new insecticidal trait should be evaluated against a diversity of non-target species, but a new trait that improves nutritional quality is highly unlikely to have any negative impact on non-target species.

A data package for a new commercial trait may cost up to US $13–18MM and take three to four years to develop, depending on the type of trait, complexity of the event and regulatory studies that are conducted.[10] And the costs and amount of time continue to increase.

16.2.8 Data Development and the Role of Good Laboratory Practices

Consistent with many other industries, most regulatory agencies base their decisions on data developed by the applicant. These studies are conducted under stringent protocols and record-keeping. Some countries require that particular studies

[10] Report on the Costs and Time for Plant Biotech Research & Development," commissioned by CropLife International, conducted by Phillips McDougall, 2012.

Table 16.3 Categories of typical biotech product safety studies required for international product approval

Methodology

- Develop methods for the synthesis of gram quantities of all novel proteins in a bacterial expression system and purification of all expressed novel proteins from that system
- Develop a method to purify small amounts of novel proteins from transgenic plants
- Demonstrate equivalency of bacterial and *in planta* produced novel proteins (e.g., activity assay, immunochemistry, amino acid analysis)
- Produce and characterize purified sample of each bacterially produced novel protein for toxicology testing
- Produce analytical standard for each novel protein for use in assay validation
- Develop and validate quantitative assays for all expressed novel proteins (e.g., ELISA, activity assay)
- Develop and validate qualitative diagnostic test for event (e.g., ELISA lateral flow strip)

Event characterization

- Describe biology of the host plant with special reference to any toxic, allergenic or weedy characteristics
- Describe method of transformation and sequence of inserted rDNA
- Describe phenotype of transformed plants (e.g., herbicide tolerance, insect resistance, drought tolerance)
- Characterize nature of insertion (e.g., Southern analysis) and determine insert/gene copy number and any molecular re-arrangements
- Describe complete nucleic acid sequence of all inserted DNA including all insert/host DNA border sequences
- Analyze sequence for novel open reading frames (ORFs) and host gene disruptions
- Determine if any novel ORFs are expressed (e.g., mRNA and protein assay) – if detected they must be treated as additional novel proteins
- Develop and validate quantitative event specific PCR-based diagnostic test based on unique insert/host border nucleic acid sequence
- Measure novel gene expression in different plant tissues over time at least five different locations representative of the typical agricultural growing environment and over at least two growing seasons

Dietary risk assessment

- Determine if there is significant nucleic acid sequence homology between each novel protein and any reported toxin
- Determine if there is significant nucleic acid sequence homology between each novel protein and any common allergen
- Investigate the significance of any homology by screening the novel protein against sera from patients with documented allergy to the homologous protein (e.g., Western blotting)
- Determine digestibility of each novel protein in simulated gastric fluid and characterize any large resistant fragments
- Determine digestibility of each novel protein in simulated intestinal fluid and characterize any large resistant fragments
- Determine heat stability of each novel protein (e.g., enzyme activity or immuno-reactivity)
- Screen each novel protein against sera from patients with documented allergy to the transformed crop (where there is a documented history of allergy to that crop)
- Conduct acute oral gavage study using a single massive dose (e.g., 500x) of each purified novel protein using mice or rats

(continued)

Table 16.3 (continued)

- Conduct 28-day sub-chronic feeding study of each purified novel protein using mice or rats
- Conduct 42-day chicken feeding study with diet incorporated grain or meal from transgenic plant
- Conduct 90-day rat feeding study with diet incorporated grain or meal from transgenic plant

Environmental risk assessment

- Evaluate any changes in phenotype that may increase the weediness potential of the transgenic plant
- Evaluate potential for, and impact of, any cross between the transgenic plant and any sexually compatible wild or weedy species present in the same environment
- Measure rates of degradation of each novel protein in a variety of soil types
- Measure rates of degradation of each novel protein in water
- Determine the effects of exposing laboratory indicator species to exposure to each novel protein by most appropriate route of exposure (e.g., purified protein in solution, leaf tissue, pollen) – species can include
    - Soil invertebrates [e.g., earthworm (*Lumbricus terrestris*), springtail (*Collembola sp.*)]
    - Aquatic invertebrates [e.g., waterflea (*Daphnia sp.*)]
    - Representative insects [e.g., honeybee (*Apis mellifera*), lacewing (*Chrysoperla carnea*)]
    - Aquatic species [e.g., trout (*Oncorhynchus sp.*)]
    - Avian species [e.g., quail (*Colinus virginianus*)]
- Analyze impact on any endangered or threatened species of any exposure to novel proteins

are conducted by public scientists appointed by the government (e.g., rodent feeding studies in China), however, this is the exception rather than the rule and most agencies lack the resources to conduct extensive and costly investigations of the safety of each new product. More often, it is incumbent on the developer to ensure the quality and accuracy of the data that is submitted in support of an application and there may be legal penalties for submitting inaccurate or false data. Increasingly, agencies require, or express a strong preference, that data be developed using internationally recognized Good Laboratory Practices (GLPs).[11]

Compliance with GLPs helps ensure the consistency and reliability of results by requiring that data developers keep detailed records that enable the audit and reconstruction of any study submitted to a government agency. While GLP compliance adds significantly to the costs and complexity of developing regulatory data, the applicant has the assurance that compliant data is likely to be viewed with high degree of credibility by regulatory agencies. That being said, non-GLP compliant data has also been used, successfully, to support applications for approval of biotech products.

[11] http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=ENV/MC/CHEM%2898%2917&doclanguage=en

16.2.9 Time Needed to Review Applications

Although regulations often state time limits for various stages of the review process, in practice these serve only as a guide. Sometimes regulatory agencies have a legitimate reason to "stop the clock" while the applicant responds to questions, but increasingly delays arise because government regulatory resources are overstretched or groups of technical experts cannot be assembled with sufficient frequency to review an increasingly large number of applications. Also, the complexity of applications has increased since many of these regulations were originally drafted. For example, those countries that regulate products comprised of biotech traits that are combined by conventional breeding, as requiring separate approvals have an especially heavy workload, because they typically review the single biotech traits first and subsequently combinations of those traits in the product containing multiple biotech traits.

It is unlikely that any agency will guarantee to meet a particular deadline, but submissions that comprise complete packages of high quality scientific data, presented in the format expected by the regulators and carefully reviewed to eliminate errors, stand the best chance of receiving more timely approvals.

However, in some cases, it is clear that politics plays a significant role in the timing of approvals. The biotech corn product identified as DAS-01507-1 (also known as TC1507 event or by the commercial name Herculex® I[12]) was co-developed by Pioneer Hi-Bred, a DuPont business (Pioneer), and Dow Agrosciences LLC (DAS) and provides protection primarily against the European corn borer. The TC1507 event contains two transgenes; an insecticidal *cry1F* gene from *Bacillus thuringiensis* (Bt) that confers resistance against lepidopteran pests (e.g., European corn borer) and the *pat* gene from *Streptomyces viridochromogenes* that confers tolerance to the herbicide glufosinate.

Pioneer and DAS filed for EPA registration in January 2000, USDA de-regulation in May 2000 and initiated a consultation with FDA in June 2000 (Table 16.4). To enable free flow of grain containing the TC1507 event they also filed for food, animal feed and environmental approvals in several importing countries including Australia/New Zealand, Canada, China, EU, Japan, Korea, Mexico, Philippines, South Africa and Taiwan, as well as cultivation applications in seed markets including Argentina, Brazil, Canada and the EU.

The USDA deregulated TC1507 in June 2001 and EPA registered TC1507 in October 2001. Canada also approved cultivation of TC1507 in October 2002, paving the way for US and Canadian sales for the 2003 growing season. Approvals from Japan and Korea were received in 2002, and approvals from other key export markets (Mexico and Taiwan) were anticipated before the 2003 harvest. Based on issued and anticipated approvals, first commercial sales occurred in the winter of 2002 for planting in the 2003 growing season in North America. All key approvals were in hand by the time the 2003 harvest entered commerce.

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[12] Herculex is a registered trademark of Dow AgroSciences LLC

Herculex I *Insect Protection* technology by Dow AgroSciences and Pioneer Hi-Bred

Table 16.4 Timing of key submissions and approvals for event DAS-01507-1 (a.k.a. TC1507 or Herculex® I)

Country	Environment import	Environment cultivation and import	Food	Feed
Argentina				
Filed		07/2001	09/2001	
Approved		03/2005	03/2005	
Australia/NZ				
Filed			05/2001	
Approved			08/2003	
Brazil				
Filed		10/2006		
Approved		12/2008		
Canada				
Filed		11/2000	11/2000	11/2000
Approved		10/2002	10/2002	10/2002
China				
Filed		04/2002		
Approved		04/2004		
European Union				
Filed	05/2001		11/2000	
Approved	Pending (Cultivation: as of July 2012)		03/2006	06/2011*
Japan				
Filed		05/2004	02/2001	12/2000
Approved		03/2005	07/2002	05/2002
Korea				
Filed			01/2001	12/2003
Approved			12/2002	11/2004
Mexico				
Filed			12/2001	
Approved			11/2003	
Philippines				
Filed	2009	05/2003		
Approved	Pending (as of July 2012)	10/2003		
South Africa				
Filed		04/2002		
Approved		12/2002		
Taiwan				
Filed			04/2002	
Approved			11/2003	
United States				
Filed		05/2000 USDA	06/2000	
Approved		06/2001	05/2001	
Filed		01/2000 EPA		
Approved		10/2001		

*At one time TC1507 was legally on the market in the EU, but had not been formally approved for feed use because under previous law it sufficed for the applicant to make a simple "notification." Under revised legislation, this simpler procedure for feed was deemed unsustainable. Dow Agrosciences and Pioneer provided the necessary studies, and EFSA agreed 1507 was safe for use in feed in June 2011 (as they had previously done for food). Since the use for food had been explicitly approved in 2006, the EU chose to amend its decision from 2006 to add the feed approval, thus leaving the original food approval in place and also making the approval for both feed and food use coterminous (they will both run out 10 years from the date of the original 2006 approval).

It is noteworthy that, while not regarded as a key import approval, a final decision for approval of TC1507 imports was not obtained from the EU until 2006, 64 months after the application was first submitted. This disparity is not unique to TC1507, but has proved a consistent feature of the EU review process. While the stipulated timeframe for review and approval of a biotech product in the EU is less than 18 months, the process typically involves multiple rounds of technical reviews followed by votes taken in committees with representations from all Member States. If applications for agricultural biotechnology products do not receive a majority vote in favor of approval, but there is also an absence of a majority against an application, the application proceeds through the process for a final approval by the European Commission.

Nowhere are the political influences on the EU review process more obvious than in applications for approval to cultivate biotech seed products in EU Member States. Since 1999, the EU operated a de-facto moratorium on the approval of biotech crops for cultivation and this was apparently lifted in March 2010 with the cultivation approval for starch-modified potato (BPS-25271-9). An examination of the timeline for the application for cultivation of TC1507 in the EU under Directives 90/220/EEC and 2001/18/EC is highly instructional in this respect.

It is well known that a number of EU Member States are vehemently opposed to the cultivation of biotech crops, thus any decision to approve the unrestricted planting of TC1507, or other biotech crops, in any country in the EU would be extremely controversial.

Over 10 years have passed since the original EU submission of TC1507, and despite multiple positive opinions from European Food Safety Authority (EFSA) confirming the safety of TC1507, there is still no final decision on the approval to cultivate TC1507 in the EU. Meanwhile corn hybrids containing the TC1507 event have been grown extensively in Argentina, Brazil, Canada, Columbia, Honduras and the US without any adverse human health or environmental consequences.

16.2.10 Renewals

A USDA deregulation is a one-time decision, but some other jurisdictions issue time-limited approvals with renewals generally between five (e.g., Taiwan) and 10 years (e.g., EU). Renewals can be straight forward, subject to no reports of significant adverse effects (e.g., Taiwan, China) or substantive requiring data gaps that have materialized over the intervening time to be filled (e.g., EU). Almost all regulatory agencies have a statutory requirement that any significant adverse findings concerning a commercial product, which might negatively impact the environment or food and feed safety, should be reported to the appropriate agency. Such reports may lead to reassessment of an approval as occurred when pollen from Bt corn plants was shown to have an adverse effect on Monarch butterflies in artificial laboratory feeding experiments. The EPA subsequently determined that Monarch butterflies in the wild would not be significantly negatively impacted by Bt corn and the registrations were maintained.

16.2.11 Regulatory Agency Fees

An increasing trend is for regulatory agencies to charge a fee to conduct product reviews. For example, Food Standards Australia New Zealand typically charges around US $10,000 dollars for review of a standard application. While such fees increase the cost of bringing a product to market, it is generally recognized that fees can help to support hiring sufficient staff to ensure reviews can be conducted in a timely manner. Hence, the agricultural biotechnology industry was supportive of fees for services by the US EPA's Office of Pesticide Programs to help achieve staffing levels necessary to meet expectations for timely reviews of applications.

16.2.12 New Technological Challenges to Regulations

Technological advances can even challenge the scope of regulation. Most biotech regulations were drafted based on examples of simple genetic modifications that altered a single trait, often combined with a selectable marker gene. As multiple biotech traits became available in the same crop, the potential arose to make combinations by breeding with already approved events to derive, for example, corn with resistance to both European corn borer and corn rootworm, as well as tolerance to glyphosate herbicide. These combinations of traits, often referred to as gene or trait "stacks" quickly became the industry standard, but revealed a lack of consensus between regulatory authorities, even within the same country, as to "if" and "how" they should be regulated.

Combinations of different traits derived by cross breeding with already approved biotech events are not regulated by USDA. The USDA regulatory process results in a determination of non-regulated status for a particular event, consequently that event and any progeny derived from it, are not subject to further regulation, even when combined with another deregulated event. However, if two different events confer resistance to different insect pests or even to the same pests are combined, the resulting stack is regulated by the EPA and a new registration is required. The same stack, would not likely be the subject of FDA consultation, but FDA may choose to review information on the composition of a stack of two previously approved events that alter a similar or related metabolic pathway.

Some countries (e.g., Japan) acknowledge that familiarity with the previously approved biotech traits that are in a stack allows for an expedited review that focuses on the potential for unintended interaction between these traits. If a stack involves modifications to complex metabolic pathways, which arguably may increase the potential for interactions, a more extensive review is required. In the case of the EU, combined traits are subjected to what amounts to a completely new review in which the data sets presented for the individual events must be repeated for the stacked trait combination, compared to the single events and the absence of any unintended interactions between them confirmed, irrespective of the mechanism of action of the modifications. Furthermore, agencies like EFSA postpone the review of the stacked

product until the single events have been fully evaluated, thereby extending the approval timelines.

16.2.13 Evolving Data and Registration Issues

Data protection is a controversial area of international regulation that initiates strong disagreements between advocates of the public "right to know" and those who seek reasonable protection for their intellectual property. Most companies will share safety information about their products, but do not want information disclosed by regulatory agencies in such a way that it can be utilized by competitors. Companies that have spent millions of dollars generating a regulatory data package do not want a competitor to be able to avail themselves of that information, free of charge. This is especially true in the new era of stacked trait combinations where one company might seek to gain regulatory approval for a stack containing a competitor's trait against the wishes of that party. This is not a problem unique to biotechnology, but exists for other regulated products like crop protection products and pharmaceuticals. Some agencies and countries (e.g., US, Canada and EU) have developed specific provisions for data protection within their regulations that provide for periods of confidentiality and/or exclusive use of the data by the technology developer. However, such provisions may not protect data from being disclosed to organizations with no commercial interest in the technology (e.g., public interest groups). In other instances, the owner of the technology must rely on provisions for protection of confidential business information that are typically developed as part of international trade agreements. Absent a claim of confidentiality, applicants can expect to see very extensive descriptions of data packages submitted for review appearing on the web sites of some agencies (e.g., Australia/New Zealand Food Safety Authority[13] and the EFSA).[14]

With the increasing development of stacked trait combinations, situations are arising in which a technology developer licenses an approved biotech event to another company, who then combines that trait with one or more of their own traits to make a stacked product. In those countries that regulate stacks, the problem then arises as to accountability for the regulatory approval for the new stack and access to any data submitted previously by the company that obtained the original approval for the licensed event. In order to protect the intellectual property of registrants, many regulatory agencies require evidence that the new applicant has the right to cite previously submitted data on the licensed event from the licensor, as a part of their application for the stacked product. Typically they request that the original registrant provide written confirmation of the licensee's right to cite their data via a

[13] http://www.foodstandards.gov.au/consumerinformation/gmfoods/gmcurrentapplication1030.cfm

[14] http://registerofquestions.efsa.europa.eu/roqFrontend/questionsListLoader?panel=GMO&questiontype=2

letter of authorization before they access the data for review. However, few countries have regulations that specifically describe provisions for data protection and access, so the legal basis for such practices is uncertain. Also largely untested, is the question of who is accountable from a regulatory perspective if a problem arises with a previously approved event after it is incorporated into another company's stacked product.

In addition to trait licensing, companies may also develop cooperative agreements to jointly develop biotech products and share research and regulatory costs. As a practical matter, the two companies may desire to obtain joint approvals for cultivation and/or import of the resulting products. This is not always possible since some regulatory agencies (e.g., EPA) refuse to acknowledge joint registrants, presumably because of concerns over accountability. There are impending issues with product discontinuation, patent expiry, access to regulatory data and maintenance of regulatory approvals. Continued maintenance of global regulatory approvals is needed to ensure the stability of the US grain export market. In addition, access to regulatory data will be necessary to enable innovation and allow for the development of stacked products containing traits following patent expiry (generics).

16.3 Stewardship

Stewardship is the responsible management of a product from its inception, through its commercial life, to its ultimate discontinuation. This life cycle requires careful attention to the safety and management of products and their market impact. Rules and regulations define legal requirements, such as, the minimum distance by which regulated biotech research plots must be separated from other crops to prevent cross pollination. The methods used by companies to ensure compliance with that requirement is a matter of stewardship. For responsible companies, however, stewardship implies not only regulatory compliance, but also the establishment and implementation of best practices that go beyond meeting minimal regulatory requirements. For most global developers, stewardship extends into such areas as supporting research on proprietary products in public research institutions; understanding how best to deploy technology and educate growers on the best way to cultivate products; and promoting sound agricultural practices that minimize environmental disturbance and market disruptions.

In general, stewardship programs comprise three elements:

1. A clear description of the objectives and endpoints;
2. Principles and desired management practices; and
3. Guides to understanding and implementing stewardship and quality management systems and processes supported by appropriate training including an audit program to identify and amend areas needing improvement.

The process of developing a new biotech crop product is long and complex with many critical points in need of stewardship attention, beginning with the identification

of genes in the laboratory. Effective stewardship requires an ongoing assessment of any risks to the environment or human and animal health posed by research and product development activities. This should commence at the gene identification stage with a thorough investigation of the properties of any gene expression products, particularly if they are identified as known toxins or found to be allergenic. If either is the case, a plan should be developed to eliminate such genes from the research program or special handling procedures should be implemented.

To produce a desired trait, genes are identified and introduced into plants through application of biotechnology techniques and tested in controlled environments, such as greenhouses. Typically many different 'events' containing the genes of interest will be created and tested. Maintaining event integrity poses a challenge in large scale screening operations, so it is critical that detailed record keeping and effective labeling practices are in place throughout the product development process. Diagnostic tests that are specific for traits and for each event are extremely valuable tools to ensure that events are correctly identified and segregated. Greenhouse operations must make certain that plants are kept separate, are individually identifiable and that strict containment measures are in place to prevent cross pollination leading to event mixing or misidentification. For example, all vents in the greenhouse should be covered with mesh to ensure that insect pollinators are kept out. Desired pollinations must be carried out according to established practices that ensure self- or cross-pollinations are not compromised and the seed produced must be harvested, labeled, inventoried and stored in a secure location. Plant materials that are no longer wanted must be disposed of in a way that ensures they are rendered non-viable and records should be kept that document their disposal.

Based on their performance, a limited number of promising candidate events are selected for field testing in small plots at one or two locations. The specific containment requirements are identified in the approval or permit obtained for conducting the trial from the appropriate regulatory agency. The institution conducting the trial must have guidelines in place to ensure compliance with those requirements. The guidelines should include instructions that specify how test entries will be checked against the permitted materials; how seed will be packed and transported to the planting site; and outline the method to verify and record receipt of the specific batches of seed provided. Before planting, it should be standard practice to re-confirm that the location of the planned trial is the same as that approved in the permit and that isolation requirements can be met. This is particularly important where isolation zones, which must be kept free of sexually compatible crops, extend across neighboring farms. For example, a neighbor may have agreed in February to plant soybeans to enable isolation of a research plot of regulated biotech corn, but two months later might change his or her mind and plant corn.

To aid compliance and provide a chain of custody that can be audited, many companies provide the research staff conducting field trials with compliance notebooks that contain information on how the trial is to be planted, monitored and terminated, as well as forms to record information such as numbers of seed received, date planted, quantity planted, observations on the emergence and growth of the crop, any observations on growth habit, disease susceptibility or agronomic anomalies. The notebooks include instructions for experimental treatments, such as herbicide applications

and serve to record harvest dates, volumes harvested and disposition of the seed as well as descriptions of how the trial was terminated to make sure no viable material remained at the site. Inspections during the following season to confirm the absence of volunteer plants are also documented. The notebooks can serve as a valuable source of information should a field site be inspected for compliance by third party auditors or government officials.

Events that perform well in the initial field trial will be planted in following years in larger plots and at more sites representative of the targeted growing environment. Those events will be introduced into elite genotypes that are the basis for current commercial products, and will be tested for compatibility with other traits with which the events may eventually be combined. Having comprehensive stewardship plans and tools in place becomes increasingly important as the scale of testing increases and the opportunity for handling and management errors increases. It is an established practice to carry several candidate events, in parallel, through the product development process until a single event is selected for commercial advancement. Advancing several events can save years on product introduction, but increases the risk of mixing events. Good stewardship procedures call for repeated testing of material at all significant hand-offs to confirm event integrity and product quality and to prevent a situation where an event, other than that which received regulatory approval, is commercialized.

A separate testing program must be initiated to develop the bio-safety data necessary to fulfill regulatory requirements and assure that products placed into commerce are safe for the environment and for humans and animals who consume them. Regulatory testing is subject to the same permit requirements as research activities, but companies may also need to comply with GLP requirements when developing regulatory data to ensure that data submitted for commercial approvals meets the quality standards of all international regulatory bodies.

At some point, candidate biotech products containing the selected events must be evaluated in plantings within grower fields to compare with current commercial products under typical cultivation conditions. It is generally impractical to do this while materials are regulated and must be planted in isolation and destroyed after harvest, so it is at this point that developers seek domestic commercial regulatory approvals.

The seed of successful new biotech products must be increased in anticipation of commercial sales. This may require several rounds of planting and harvest. The seed industry has developed stewardship and quality practices over the years to increase seed while maintaining genetic integrity. These practices involve the use of isolation distances to prevent the introductions of pollen from commercial crops into the seed crop. With the commercial deployment of several biotech traits and multiple events in crops, the need for testing programs to confirm product integrity is critical. The conventional seed that is sold as not containing biotech traits must be tested to confirm the absence of transgenic events. Where biotech seed is sold as containing a specific event, it is important to confirm that it has not been compromised with unwanted events that would give rise to event combinations that do not have regulatory approval.

Regular auditing is an essential part of any effective stewardship program. The primary function of audits is to identify weaknesses in the systems, recommend corrective actions and identify opportunities to improve protocols aimed at trait purity. Audits also serve as educational opportunities. Auditors become very experienced

in understanding which systems represent best practices and identifying those systems that could be improved upon, even though they may still be in compliance. Following an audit, the company must be committed to implementing the auditor's recommendations and have a mechanism in place to confirm corrective actions were indeed implemented.

16.3.1 Excellence Through Stewardship®

Technology developers have a great deal of experience in compliance and stewardship practices and have a good record of operating within the regulations. However, there have been a few instances where materials that did not have commercial regulatory approvals have found their way into commerce. While no adverse safety issues have resulted from these unapproved releases, the incidents demonstrated there was room for improvement. In response, the agricultural biotechnology industry coordinated an initiative to promote the global adoption of stewardship programs and quality management systems for the full life-cycle of biotechnology-derived plant products within the Excellence Through Stewardship® (ETS) organization.[15] The objectives of ETS are to promote the responsible management of plant biotechnology, primarily by developing and encouraging implementation of product stewardship practices and by educating the public about those practices.

The principles espoused by the ETS program are described as:

1. Defining and documenting stewardship programs and quality management systems designed to achieve the above-described objectives for the full product life cycle.
2. Implementing a third-party audit process that follows approved Excellence Through Stewardship® audit protocols and verifies that stewardship programs and quality management systems are in place.
3. Including stewardship and quality management requirements, practices or specifications in applicable contracts and agreements involving plant biotechnology with third parties that are consistent with the Stewardship Objectives, Principles and Management Practices.
4. Reaching out to others involved in the development and production of biotechnology-derived plant products to promote stewardship programs and quality management systems.
5. Engaging others in the food, feed, fuel and fiber value chains to promote stewardship programs and quality management systems.

The ETS organization has published a number of guides aimed at developers to provide direction on how to design and implement stewardship and management practices across the industry and at all stages of the product lifecycle.

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[15] http://www.excellencethroughstewardship.org/

- The Guide for Stewardship of Biotechnology-Derived Plant Products; (http://www.excellencethroughstewardship.org/LinkClick.aspx?fileticket=1bxJGf1Odcs%3d&tabid=84)
- The Guide for Maintaining Plant Product Integrity of Biotechnology-Derived Plant Products; (http://www.excellencethroughstewardship.org/LinkClick.aspx?fileticket=P4qw2mlAeLc%3d&tabid=84)
- The Guide for Product Launch Stewardship; (http://www.excellencethroughstewardship.org/LinkClick.aspx?fileticket=ppgyTABguQs%3d&tabid=84)
- The Guide for Incident-Response Management of Biotechnology-Derived Plant Products; (http://www.excellencethroughstewardship.org/LinkClick.aspx?fileticket=b3dc1lQkR7M%3d&tabid=97)
- The Guide for Product Discontinuation of Biotechnology-Derived Plant Products; (http://www.excellencethroughstewardship.org/LinkClick.aspx?fileticket=13xwiedNrM%3d&tabid=84)

The ETS organization also provides developers with access to third-party audits by ETS qualified auditors.

The ETS program is aimed at all companies and institutions that are involved in the development and/or commercialization of biotech plant products and is promoted through trade associations such as the Biotechnology Industry Organization (BIO) and CropLife International (CLI). Membership is open to all interested parties in the private and public sector. At the present time, the regular members comprise all the major biotechnology providers as well as some emerging companies. It should be noted that ETS is not the only stewardship program utilized by technology developers. ETS is often combined with other programs such as ISO, GLP or other quality management systems to build more comprehensive stewardship and compliance programs.

16.3.2 Insect Resistance Management (IRM)

Stewardship includes helping growers adopt the best cultivation practices for new products and comply with any unique regulatory requirements to maximize the durability of valuable biotech crops. An early concern arising from the development of insect resistant crops based on the incorporation of *Bacillus thuringiensis* (Bt) insecticidal proteins into plants was the potential evolution of resistance leading to the loss of this biological means of pest control, not only for production agriculture, but also for organic growers for whom the use of Bt is an important insect control method. Devising a method to significantly delay or even prevent the onset of resistance to Bt in target populations was a major concern for the EPA as they considered the first registrations for Bt cotton and Bt corn. EPA, academia and industry worked together to develop an insect resistance management (IRM) plan that required a part of a grower's crop acres to be planted to non-Bt containing crop as a refuge from which susceptible insects would emerge and mate with any resistant survivors from the Bt crop to minimize the potential for resistance development. This represented an entirely novel concept for growers who were used to maximizing production on

all their acres and were initially unsympathetic to the idea of leaving some of their valuable crop unprotected.

Under the auspices of a trade group of Bt corn technology providers known as the Agricultural Biotechnology Stewardship Technical Committee (ABSTC), industry undertook a stewardship program to educate their sales forces and growers as to the purpose and long-term benefits of refuges and also to assess compliance by customers. These Compliance Assurance Programs (CAPs) subsequently became a conditional requirement of product registrations, which also placed some additional responsibilities for maintaining product durability upon technology developers including an obligation to randomly assess the level of resistance to Bt in field collected samples of pest populations and to establish a system to receive and investigate reports of product failures that might be early indicators of resistance development. The objective of the monitoring program operated by the technology providers is to identify potential field resistance before it becomes a widespread problem. Numerous academic and government agency experts advise the ABSTC on appropriate monitoring programs, and ABSTC continually evaluates the program to reflect the latest research and product use.[16]

To help growers understand the rationale for refuges and how to plan and plant them for maximum effect, the ABSTC members produced Product Use Guides (PUG) specific for each trait (e.g., Herculex I).[17] Growers who purchased a Bt product received a copy of the appropriate PUG and a Technology Agreement that stated the grower agreed to implement an IRM stewardship program as specified in the PUG and authorized company representatives to make random on-farm visits to assess compliance with the program.

The CAP also outlined consistent standards developed by EPA and the companies to respond to growers who did not follow the IRM requirements to bring them into full compliance. These actions included:

- Notifying the grower by letter of IRM compliance deviations.
- Conducting a compliance assistance visit with the grower prior to planting in order to assist the grower in planning and implementing a proper IRM program.
- Conducting a compliance assessment visit with the grower the following growing season to assess IRM compliance.
- Providing the grower additional IRM educational materials.
- Denying access of Bt corn to growers who have been out of compliance in multiple years.

There are significant pressures on growers not to plant a complete refuge, especially in years when corn prices are high. Moreover, compliance was straightforward when products contained only one type of trait and one or two refuge plans satisfied all requirements. Today, there are products with different modes of action directed at the same pest that enables refuges to be smaller in size and these co-exist with more established products requiring larger refuges. The grower is faced with more decisions about refuge planting that may result in some confusion about what

[16] Agricultural Biotechnology Stewardship Committee, A Detailed Description, Dec. 2011.

[17] http://www.pioneer.com/CMRoot/Pioneer/usa/agronomy/insects/pugs/hx1.pdf

the refuge requirements are for each product. The seed companies have increased their education efforts to deal with this added complexity, but ultimately the goal is to have the refuge "in the bag" through the use of multiple Bt events with different modes of action and seed blending, thus reducing and eventually eliminating the need for a grower to plant a separate refuge.

16.3.3 Stewardship and the Academic Sector

Academic scientists represent important partners in stewardship programs since they often have specialized knowledge and resources and because their academic standing enables them to investigate issues and publish results independently. It is true that industry pays for much of this research, so in the eyes of some, the results are never completely independent. However, academic researchers invariably insist on maintaining their freedom to publish results, despite the fact that they have received funding from a commercial entity. Responsible companies accept that condition, providing confidential business information is not disclosed.

Another example of academic support for biotech stewardship is to be found in the area of animal nutrition. Regulatory agencies frequently require animal feeding studies to assess the safety of new biotech traits. These studies are usually conducted in rodent and avian laboratory model species. With the large scale introduction of biotech crops into the animal feed chain, some farmers and companies responsible for raising large numbers of animals such as beef and dairy cattle, pigs and chickens questioned if the new biotech forage, grains and oilseeds would have the same nutritional value in their feeding operations as traditional feedstuffs. To address those questions, technology developers contracted with academic experts in animal nutrition to conduct carefully controlled feeding experiments with these large animal species to determine if the nutrient value of new biotech products was the same as their traditional counterparts. Many independent studies confirmed that indeed no differences exist in nutritional value. For example, studies of dairy cows, beef cattle, pigs and chickens that were fed similar, balanced diets containing biotech TC1507 corn or traditional corn showed no significant differences in animal performance.[18] Biotech products are now readily accepted as animal feedstuffs throughout the US and in many parts of the world.

16.3.4 Stewardship and Global Trade in Agricultural Commodities

As described previously, the global trade in agricultural commodities means that before an approved biotech crop can be grown on a wide scale domestically, there must be the appropriate approvals in place to allow the grain from that biotech crop

[18] http://www.agbioworld.org/biotech-info/articles/agbio-articles/GMfeedsafetypapers.html

to enter key export markets. Until those approvals are in place, grains from that biotech crop must be kept separate (grain channeling or identity preservation) from the pool of grain destined for export.

A small number of grain handling companies account for most of the US and Canadian grains and oilseeds traded and moved around the world. While generally supportive of the rapid adoption of biotechnology by American farmers, the grain trade is concerned about the challenges of compliance with international regulations governing movement of these products. As the entities responsible for delivering commodity grains and oilseeds, they have to deal with the consequences and costs if a shipment is rejected when it arrives at port because it contains biotech products that lack the appropriate approvals. Consequently these traders have a powerful influence on technology developers, insisting that the developers obtain approvals for their new biotech crops in all key export markets before they will purchase grains and oilseeds from growers planting those biotech crops. (See: North American Export Grain Association (NAEGA) Statement on Biotechnology).[19] Growers can also make choices about which biotech varieties and traits to plant on their farms, while maintaining flexibility in the final disposition of the crop. If there is uncertainty about whether particular biotech crops will be accepted at the local grain elevator, many US and Canadian farmers will avoid planting those biotech crops, even though they have all the approvals for domestic cultivation.

16.3.5 Attempts to Segregate Products

Some developers have implemented a stewardship system often referred to as "grain channeling" to assure that domestically produced grains from biotech crops approved for domestic cultivation, but which lack all the necessary import approvals, do not enter the commodity stream, but are retained on farm or used locally. This is particularly challenging because of the way grain is freely traded and transported prior to its eventual end use. An early attempt to segregate a biotech crop by use and require that it was only planted as an on-farm animal feed showed how problematic such segregation is in practice. In this case, the grain channeling led to recalls of food products containing corn products that tested positive for the biotech trait known as Starlink™ and led to international concern over the safety of US corn causing trade disruptions.[20] It is estimated that it took at least five years to remove all traces of the biotech trait in question from the US commodity grain supply.[21] As noted previously, there have been rare instances where material that was never intended to be released commercially has found its way into commerce. The source of these problems is not always clear, but unwanted cross pollination is a significant

[19] http://www.naega.org/images/biotech.pdf

[20] StarLink™ corn was developed by Aventis CropScience.

[21] http://archive.gipsa.usda.gov/reference-library/bulletins/pn10-10.pdf

risk with a wind pollinated crop such as corn. Since soybeans are almost exclusively self pollinated, the risk of unwanted cross pollination is virtually non-existent, but inadvertent mixing of seed lots and grain batches remains a potential risk for all biotech crops.

Channeling can be successfully implemented, but only where there are rigorous identity preservation practices to prevent mixing of grain or oilseed products. Also, the cost of segregating materials from the commodity supply can be cost prohibitive, except where the biotech crop has significant added value (e.g., specialty food uses).

16.3.6 Testing for Unapproved Events

As a result of potential concerns about biotech grain mixtures, some key importing countries have adopted very rigorous purity standards for imported commodities, often demanding the complete absence of any unapproved events (zero tolerance), even though their presence in commodity grain shipments may be infinitesimal and unintentional. This has generated intense interest in technology for the rapid identification of biotech events so that grain shipments can be tested at each stage of handling and transportation to confirm, with reasonable certainty, that events without all the necessary approvals are absent. Some jurisdictions (e.g., EU, China) require that the applicant deposit a validated assay capable of identifying the specific event and sometimes a quantity of appropriate reference materials, to enable enforcement actions. Current technology based on simple and rapid immunochemical tests (dip-sticks) can detect the presence of a novel protein characteristic of a particular event with a sensitivity of about 0.1 %, but more complex and time-consuming assays based on polymerase chain reaction (PCR) technology can lower the detection limit to 0.01 %. These PCR-based methods can distinguish between different events that contain similar proteins, based on the sequence of the junction between the recombinant DNA insert and the plant genome (border sequence). Companies typically make event-specific detection methods available when their proprietary traits are commercialized. As a practical matter, however, these sensitive and highly specific assays take too long to confirm the absence of unapproved events. This often means that batches of biotech grain must be segregated and held for several days before they can be added to the commodity supply or diverted for domestic use, which adds to the operator's costs.

16.3.7 Biotechnology Industry Organization Product Launch Stewardship Policy

The Biotechnology Industry Organization (BIO) is a trade association that represents organizations involved in biotechnology in the pharmaceutical, industrial and agriculture industries. The Food and Agriculture section of BIO encourages all

members to develop product launch stewardship policies and practices consistent with its Product Launch Stewardship Policy statements and guidance.[22] Companies should determine if their product is intended for commodity use or special use, conduct a market and trade assessment appropriate to that product, develop management plans and engage key stakeholders regularly regarding market and stewardship plans.

In the case of biotech crops intended for the commodity grain channels, the market and trade assessment must include an analysis of any regulatory requirements that exist in those countries that import the grain. Regulatory requirements must be met and appropriate approvals must be obtained prior to commercialization in key markets that have science-based, functioning regulatory systems in place.

Crops are now being developed using biotechnology to intentionally modify the composition or functionality of the grain or grain product. BIO has developed additional guidance for these 'special use traits,' which are not intended to enter the commodity grain stream in significant quantities.[23] In addition to the stakeholder engagement and stewardship activities for commodity crops, it is critical to understand the potential for significant unintended processing and functional or compositional negative impacts a special use trait product may have on other processes or products of stakeholder concern. BIO member companies agree to a thorough market and trade assessment of the trait and crop and, on a case-by-case basis, develop relevant management, mitigation and incident response plans appropriate to the potential for significant unintended impacts that are identified. Key to the success of these activities is appropriate outreach and communication throughout the value chain. An example of successful stakeholder outreach is found in Pioneer's regulatory approval process, product introduction and careful stewardship of the Plenish™ high oleic soybean product[24] (See *Case Study: Plenish™ High Oleic Soybean Stakeholder Outreach, page 369.)*

16.3.8 The Challenge of Establishing Practical Thresholds

A solution to disruption in trade caused by the unintended presence of small quantities of transgenic events that are not approved in the importing country is the establishment of practical thresholds that allow a small amount of the material to be present without affecting the safety, function or movement of the commodity. The concept of establishing thresholds that permit the presence of trace amounts of materials that do not compromise the safety or functionality of a product is very well established. For example, countries establish tolerances for such things as pesticide residues which are likely to be present in grains, fruits and vegetables.

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[22] http://www.bio.org/letters/Product_Launch_Stewarship_12_10_09.pdf

[23] Annex 2 of http://www.bio.org/letters/Product_Launch_Stewarship_12_10_09.pdf

[24] Plenish™ is a trademark of Pioneer Hi-Bred.

Attempts to establish thresholds for unapproved and/or undeclared biotech events in commodity grains and oilseeds have been met with strong resistance. This reflects, in part, a high level of misinformation about the safety of biotech crops in many countries. Despite the provision of factual and science-based information on the safety of biotech crops, it is difficult for governments to acknowledge that any level of unintended presence is safe, thereby enacting a de-facto zero tolerance.

It should be noted that there is also a fundamental difference between a chemical residue in a batch of grain and the presence of a seed with a biotech trait. The chemical residue can never multiply, but a single kernel of corn is capable of growth and reproduction and, if placed in the right environment, can theoretically pass transgenes to local crops and related wild species through cross pollination. Invasion and establishment in the native environment is an unlikely outcome for most of our highly domesticated crops that are reproductively compromised, but it remains a concern that complicates any discussion on acceptable thresholds.

16.3.9 Adventitious Presence and Low-Level Presence

Adventitious Presence (AP) and Low-Level Presence (LLP) refers to low level, unintentional introduction of plant material from plants developed using modern biotechnology, that has been through a full safety/risk assessment in one or more countries, but not in the country of import.[25] Specifically, AP refers to low level unintentional introduction of biotech-derived plant material in ***seed*** that has been through a full safety/risk assessment in one or more countries, but not the country of import. And LLP refers to low level unintentional introduction of biotech-derived plant material in ***grain/feed*** that has been through a full safety and risk assessment in one or more countries, but not the country of import.

As a practical matter, by implementing good stewardship practices in the production of seed material, biotech crop providers are capable of keeping the level of unintended events at a very low level. The seed industry believes it is possible to regulate a low-level presence (LLP) that is scientifically sound and will facilitate global trade. Such regulation would tightly control and minimize the movement of genes or gene products that have not been scientifically assessed for adverse effects on human or animal health. However, where a gene and/or gene product, or a biotech event, has been reviewed and approved by a responsible regulatory body and found to meet applicable regulatory safety standards, then an agreed amount of

[25] AP and LLP is an unavoidable reality of plant biology, seed production and the distribution of commodity crops. There are a number of factors that contribute to commingling: pollen flow; volunteerism; mixing during harvesting, transport, storage and processing; human error; and accidents can all play a role in adventitious presence. While adventitious presence can be minimized, as a practical matter it cannot be eliminated entirely and is not unique to conventional crops or crops enhanced through biotechnology. AP and LLP does not necessarily compromise food safety.

LLP of that gene or biotech event should be accepted in seed products within existing criteria for seed purity or commodity grain shipments and should not be a cause for rejection.

16.3.10 Trait Retirement

Technology developers constantly strive to improve the performance of their crops and the traits that provide enhanced traits and improved yield. At some point, older traits may become non-competitive against newer technologies and will be retired from the marketplace. What used to be a simple matter of stopping the sales of a particular hybrid or variety, is more complicated in the world of biotech crops. Good stewardship requires that developers create a plan to phase out the sale of biotech products containing older events, but maintain the global regulatory approvals long enough for the grain produced from hybrids and varieties containing the trait to work its way out of the commodity stock. As seen by the experience with unapproved biotech events in the grain channel, this can take several years if significant acreages were planted with the biotech trait.

16.4 Stakeholder Outreach

To assure timely and favorable trait approvals, technology developers expend considerable efforts to reach out to stakeholders and provide information to explain the benefits of their products, how they will be responsibly managed and to allay any concerns. The aim is also to develop a long-term relationship with key strategic partners, not just to obtain regulatory approvals. It is also important that they work together to develop appropriate stewardship programs, deal with risk management in a cooperative and rational manner, and create interest and support for future applications of the technology, as well as promote public acceptance.

Stakeholder outreach in agricultural biotech focuses on four main objectives:

1. Obtaining regulatory approval for transgenic products;
2. Advancing market acceptance of the technology;
3. Reinforcing (or establishing) a developer as a credible technology provider and an active participant in the industry; and
4. Minimizing the potential for disruption of the grain trade.

16.4.1 Best Practices for Strategic Stakeholder Engagement

Responsible developers aspire to core values that demand integrity, high ethical standards and are fair and respectful in their dealings with all stakeholders. They strive to produce the best products, deal honestly and fairly with all stakeholders,

Fig. 16.5 Food and agriculture value Chain; segments and influencers

promote their products vigorously without misrepresentation and provide reliable information to customers and other stakeholders to assist them in making the best possible use of new technology and products while maintaining a strong focus on the environment and conservation of biodiversity.[26]

16.4.2 Identifying Stakeholders

The first step in any program is to identify the key stakeholders. Each stakeholder in the chain has a different requirement for information about new food/feed products. Responsible technology developers will ensure each stakeholder is engaged at the appropriate time and that the stakeholders' questions and concerns are heard and addressed before a new product is introduced. For some, it may require providing detailed technical information, while for others it is important just to be consulted.

Undoubtedly for the seed industry, the voice of the grower is very important in making decisions about what, how and when to bring products to market. Beyond the grower, who will actually plant a new agricultural biotech crop, there are many stakeholders along the entire food value chain—from seed companies that license the trait to food manufacturers and consumers. Developers that ignore their stakeholders along the entire food value chain (Fig. 16.5), do so at their peril.

Regulatory agencies are key stakeholders for biotech trait developers since they grant product approvals and control access to the market. An agency decision to approve or reject is based on safety of a product, but agencies are also subject to other sources of influence in making a final determination including the opinions of policy makers, academics, public interest groups and the judiciary. The decision to approve a product is much easier if an agency knows that stakeholders are supportive of the decision. Serious reservations expressed by influential groups will often be reflected in delayed decisions, time limited approvals, onerous restrictions on use or even rejections.

The food and agriculture value chain represents many different organizations, trade associations, interest groups and independent entities. For example, academics are typically represented by learned societies such as the Crop Science Society of America and the Entomological Society of America, while technology developers and seed companies look to trade associations such as BIO, CLI and the American Seed Trade Association (ASTA) to represent their interests. Farmers are represented by groups such as the National Corn Growers Association (NCGA), American

[26] "The Long Look," Pioneer Hi-Bred, a DuPont Business, 1952.

Soybean Association (ASA), American Farm Bureau Federation (AFBF) and National Farmers Union (NFU), as well as an array of state associations.

Similarly, there are trade groups such as the National Cattlemen's Beef Association and Pork Producers Council, as well as groups who represent the feed industry and the commodity grain industry, such as the National Corn Refiners Association (NCRA), National Oilseed Processors Association (NOPA) and the North American Export Grain Association (NAEGA). Stakeholders also include distributors such as the Grocery Manufacturers Association (GMA), as well as the multinational food manufacturing and retail companies that play a key role in delivering products to the consumer.

Opinion influencers also include organizations that claim to represent consumer and environmental interests such as Center for Science in the Public Interest, Center for Food Safety and Union of Concerned Scientists. There are also a number of global organizations that may be appropriate to consider in an outreach program including The World Bank, OECD, WHO, UNFAO, Natural Resources Defense Council, World Resources Institute, Environmental Defense Fund, Business for Social Responsibility, World Business Council for Sustainable Development, The Nature Conservancy and World Wildlife Fund.

Government stakeholders, beyond the regulatory agencies, include the US Departments of Justice, Commerce and State and also include the Office of the US Trade Representative and the US Agency for International Development. The media are also important stakeholders and are part of any outreach plan, but are outside the scope of this discussion.

It is important to appreciate the interconnectedness between different stakeholders in the chain. The push and pull for technology acceptance can be initiated at any part of the value chain and may result in major road blocks. For example, foods containing or derived from biotechnology crops must be specifically labeled in Europe. Having raised public concerns about the safety of foods derived from biotech crops, certain public interest groups were then able to bring pressure to bear on supermarkets to withdraw these food products. One major grocery chain succumbed, and shortly thereafter, other retailers followed suit. The result was that in many European countries there was no significant outlet for foods derived from biotech crops, and imports of commodity grains and oilseeds from the US destined for food use virtually ceased. European food processors were forced to source and import materials certified as derived from non-biotech (conventional) crops, largely from South America or to substitute other ingredients, where possible. As South American countries increasingly adopted agricultural biotechnology, the price of such identity preserved non-biotech materials have increased to the point where today, some European retailers are considering re-introducing foods containing biotech materials.

While no amount of outreach would likely influence those public interest groups who are vehemently opposed to the technology, better industry outreach to the retailer community arguably might have prevented products being pulled off store shelves and non-GM status becoming a competitive marketing strategy, which did not result in superior products.

Case Study: Plenish™ High Oleic Soybean Stakeholder Outreach

Plenish™ high oleic soybeans were the first USDA-deregulated biotech soybean product with direct consumer benefits.[27] *Plenish high oleic soybeans provide enhanced oil with the highest oleic content in soybeans under commercial development, meeting food industry needs and consumer demand for a soy-based trans fat solution with increased functionality and 20 percent less saturated fat than commodity soybean oil. There are also bio-based industrial applications for high oleic soybean oil, including its use as a renewable and environmentally friendly option to petroleum-based products in a number of industrial applications.*

A product of Pioneer Hi-Bred, a DuPont business, Plenish is an excellent example of a biotech trait that required extensive stakeholder outreach along the entire food value chain throughout the deregulation process because of the opportunities it provides growers, processors, food manufacturers and consumers. For that reason, Pioneer began a coordinated outreach effort to key stakeholders such as the ASA as early as 2007. In 2008, Pioneer expanded its educational outreach on the benefits and opportunities of the product to other key groups including NCGA, NGFA, NAEGA, NOPA, AFBF, NFU, GMA and numerous other organizations to answer technical questions about the product and outline the timeline for the regulatory review

In addition to facilitating the deregulation of the high oleic soybean trait, early outreach by Pioneer has created demand and facilitated use of the product. To date, Pioneer has partnered with more than 205 major food companies, oil processors and industrial users in advance of commercialization to test Plenish high oleic soybean oil. Testing has included shelf life, fry life, baking performance and industrial applications to demonstrate the stability of the oil and create downstream demand for the product. Demand from oil processors, manufacturers and consumers provides incentive for growers to produce the product, which requires identity preservation and grain channeling contract requirements.

Plenish high oleic soybeans were deregulated in the US in 2010, and received prior approval in Canada and Mexico. At the time this book went to print, Pioneer was still awaiting additional import approvals, with commercialization anticipated in 2012 (upon full regulatory approval and field testing). Pioneer will continue to work closely with the supply chain on the stewardship requirements of growing, harvesting, handling and marketing Plenish high oleic soybeans to ensure the product does not prematurely enter a market before it has been approved for import in key markets.

16.4.3 Planning Stakeholder Engagement

A successful outreach program is built on a number of principles: conducting trade and market assessments for potential products, identifying the right stakeholders; engaging them at the right time and with appropriate intensity; and delivering the right message, while maintaining realistic expectations.

Most large developers devote significant resources to understanding the scope and interests of their stakeholders. They maintain databases of potential stakeholders, their senior staffers and stated positions on current issues as well as identifying a key internal "owner" responsible for maintaining the company's relationship with the organization and a history of previous contacts and outcomes. In some instances,

[27] http://www.emilywaltz.com/Oleic_soybeans_Aug_2010.pdf

the relationship is formalized through membership, participation or supported by financial contributions to the activities of the organization, such as sponsoring sessions at annual meetings.

In any outreach plan, the stakeholders must be prioritized or "mapped" according to their interest and relevance to current and future projects. For example, stakeholder organizations that are deemed to have little interest or capacity to engage and influence outcomes may only require monitoring. They receive general information and feedback is solicited. Another group may be influential on certain issues, but not those of current interest. Here the objective is to maintain an on-going dialogue and provide support as appropriate to maintain a relationship for future engagement.

Other groups may be important influencers on key issues, but do not have a relationship with the company. Initially the plan might be to inform, consult and solicit feedback on new issues, as well as provide access to decision makers in the organization and identify resourcing opportunities.

Ultimately, for those organizations that are key to successful product approval and deployment, the goal is to establish a strategic partnership with a long-term perspective—not to simply ask for one-off favors when there is a product to approve or a problem to resolve. Developing a long-term relationship requires regular meetings to update on the progress of all relevant projects, access to senior company decision makers and an open and frank dialogue as to the implications of new technologies and products that, over time, will establish a level of trust between parties and enable a constructive working relationship.

Timing is also critical to achieve an effective interaction with a particular stakeholder. The developer must aim to control the information flow and avoid situations in which, for example, key stakeholders learn of a new product application or impending problem through a third party. Equally, it is important not to engage busy organizations with limited resources in discussions that are not related to their current interests.

Each stakeholder in the chain has a different requirement for information about how new agricultural biotechnology products will influence existing systems or introduce new opportunities. In designing an outreach plan for a new soybean with improved oil profile for human nutrition, the ASA would be a critical stakeholder. However, the NCGA should be kept informed of the commercialization plans for the soybean product, because soybean is a rotational crop with corn in the Midwest. Whatever the level of engagement, the aim should be to answer their questions and meet their specific informational needs.

A responsible technology developer will ensure each stakeholder receives information that is relevant to their concerns and interests. A typical outreach plan will call for a number of different communication tools including, but not limited to, a general description of the product and how and where it will be used (both written and oral presentations); a specific information piece that anticipates questions relevant to each stakeholder or group of stakeholders; a technical package describing the nature of the product, how and when it will be deployed; product safety

information; a PUG or other examples of stewardship materials to be provided to customers; and an update on current and planned regulatory approvals.

Many of the stakeholders in the US and Canadian value chains share broadly the same interests as technology developers and are generally supportive of the introduction of new crop technologies. Some, however, may have significant reservations. The challenge is to understand and address stakeholder concerns to increase their support, or at least reduce the likelihood of objection, to product approvals or policy changes.

In seeking to influence opinion within a stakeholder organization, a company must analyze the motivations behind the current position and the organization's ability to change positions. Questions to ask include, but are not limited to: What information does this organization need? What are their concerns? Who are their stakeholders? Are there people of influence within the organization who are amenable to considering a change in position? Are there others within the organization who would likely support the change and who else might influence their decision? What are the rewards and benefits to the organization of such a change and are they getting the appropriate information and answers to promote the change?

Another key decision is when to operate as an individual entity and when to work with industry partners to achieve a desired outcome. Generally companies work independently on individual product approvals, but when it comes to influencing regulatory policy outcomes or terms and conditions of product use that impact all interested parties similarly, working collectively through a trade association can be more effective. It is extremely important that such cooperative efforts between companies are conducted within strict guidelines to avoid violating antitrust laws that prohibit anti-competitive conduct.

Even a well executed outreach plan does not guarantee success. Ultimately trade organizations and NGOs represent the interests of their members, and if a developer's aspirations conflict with those members' interests, then obtaining support for a project is unlikely. Nevertheless, the engagement will likely prove constructive in the long run should increase respect for each other's points of view and provide valuable insights into priorities and interests in the value chain that might shape future research and product design or altered policy positions.

16.4.4 Advisory Panels

Increasingly, companies are establishing third party advisory boards or panels to provide external insights and guidance on new projects during development testing and commercialization.[28] Panel members are selected for their broad experience in a wide diversity of food and agriculture-related issues and can represent certain stakeholder interests.

[28] http://www2.dupont.com/Biotechnology/en_US/advisory/index.html

Panels often include members who will challenge accepted ideas and may represent groups that are critical of the technology. Members typically enjoy a level of access to confidential business information and senior leadership that would not be available in any other venue. Panelists are generally appointed for a fixed time period and alumni serve as valuable resources to address specific questions, since they develop an in-depth knowledge of the company, its pipeline, its objectives and policies.

16.4.5 In Summary

This chapter describes how agricultural biotechnology has advanced, impacting countries, consumers and growers globally. Successful deployment of new agricultural biotechnology products not only depends on compliance with applicable regulations, but also requires effective stewardship programs to manage a product from inception through its commercial life to its eventual discontinuation and pro-active stakeholder outreach to all interested parties to promote acceptance in the marketplace. Failure to execute effectively in any of these areas puts successful product introduction and continued presence in the marketplace at risk.

Regulatory compliance is increasingly complicated, as the number of countries with regulations governing agricultural biotechnology products increases. The global marketplace for commodity grains and oilseeds produced in the US and Canada requires that technology developers have an in-depth knowledge of the patchwork of international regulations governing the cultivation and export/import of commodity grains and oilseeds, as well as the technical and financial capacity to successfully complete an ever-increasing list of sophisticated regulatory science studies required to demonstrate environmental, food and feed safety in all necessary jurisdictions. From a scientific perspective, it is an opportunity to revisit regulatory harmonization—especially for food and animal feed safety, and for cultivation in comparable environments, thereby leveraging expertise and streamlining commercialization of biotech crops without compromising safety.

Anticipating data requirements and dealing with asynchronous approvals in key export markets represents two of the biggest challenges facing developers of new improved seed products. To prevent the unintended release of commercial candidates before all key regulatory approvals are in place, which could lead to significant disruptions in international trade, responsible developers implement stewardship programs to insure the genetic identity and containment of each candidate event. Before the commercial launch of each new product, stewardship plans must be put in place to comply with any terms and conditions placed on an approval, such as mandated crop management practices to delay the development of insect resistance, as well as voluntary educational programs to insure growers are aware of any special cultivation or handling regimes necessary to insure they derive maximum benefit from new technology.

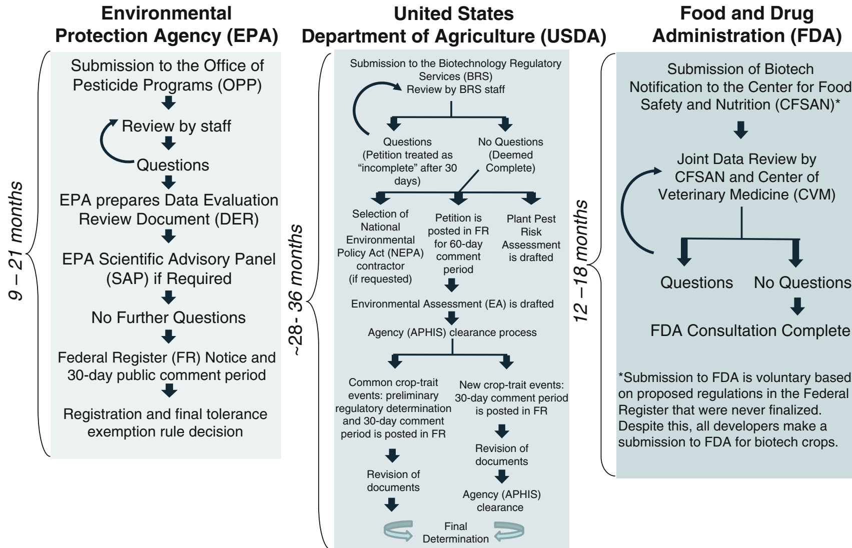
The agricultural biotechnology industry is still relatively young. Aspects of the technology are unfamiliar and may even be controversial to some. However, the technology continues to advance with no lapse in the strict regulatory scrutiny and an unprecedented safety record of biotech crops that have been commercialized. Large areas in North and Latin America routinely grow biotech crops and harvested grains from these crops are imported and used globally. Asian and African countries are also beginning to grow biotech crops, and this is likely to increase as growers realize the benefits of biotech crops applicable in their geographies. Regulatory resources will need to be multiplied to deal with the expected growth of new biotech products.

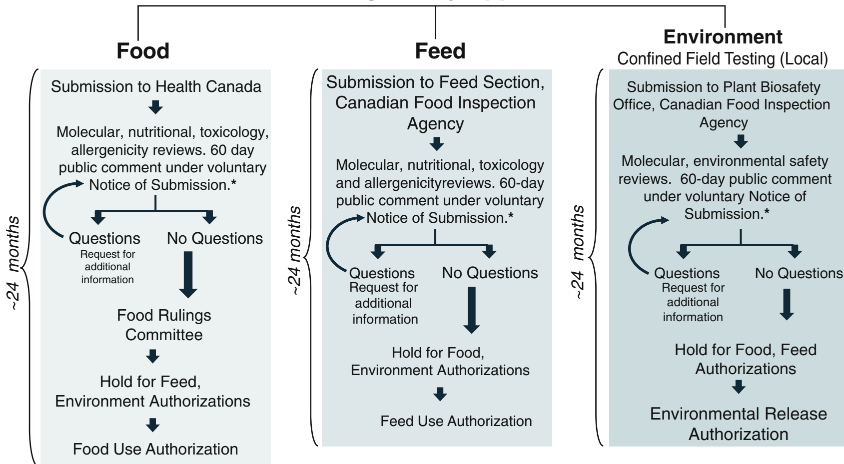
Stakeholder outreach to all interested parties is critical to promote acceptance in the marketplace. Stakeholder outreach is not simply aimed at promoting regulatory approval, but also advancing market acceptance of the technology and positioning the developer as a responsible technology provider and steward and a credible source of information on the product. An effective stakeholder outreach program establishes ongoing relationships with critical stakeholders to keep them apprised of recent developments, progress and challenges, building trust and support that can be useful throughout the global deregulation process.

16.5 Appendix

Regulatory Approval Systems of the United States and Canada and Two Key Jurisdictions for the Cultivation and Importation of Transgenic Crops.

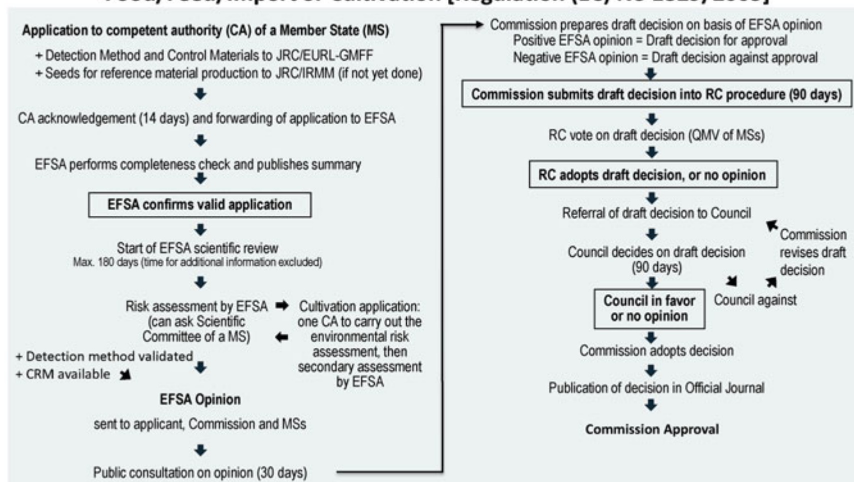
- United States
- Canada
- European Union
- Japan


United States
Environmental Protection Agency (EPA)
Submission to the Office of Pesticide Programs (OPP)
Review by staff
Questions
EPA prepares Data Evaluation Review Document (DER)
EPA Scientific Advisory Panel (SAP) if Required
No Further Questions
Federal Register (FR) Notice and 30-day public comment period
Registration and final tolerance exemption rule decision
9 – 21 months
United States Department of Agriculture (USDA)
Submission to the Biotechnology Regulatory Services (BRS)
Review by BRS staff
Questions (Petition treated as "incomplete" after 30 days)
No Questions (Deemed Complete)
Selection of National Environmental Policy Act (NEPA) contractor (if requested)
Petition is posted in FR for 60-day comment period
Plant Pest Risk Assessment is drafted
Environmental Assessment (EA) is drafted
Agency (APHIS) clearance process
Common crop-trait events: preliminary regulatory determination and 30-day comment period is posted in FR
New crop-trait events: 30-day comment period is posted in FR
Revision of documents
Revision of documents
Agency (APHIS) clearance
Final Determination
~28- 36 months
Food and Drug Administration (FDA)
Submission of Biotech Notification to the Center for Food Safety and Nutrition (CFSAN)*
Joint Data Review by CFSAN and Center of Veterinary Medicine (CVM)
Questions
No Questions
FDA Consultation Complete
*Submission to FDA is voluntary based on proposed regulations in the Federal Register that were never finalized. Despite this, all developers make a submission to FDA for biotech crops.
12 –18 months
Source: Pioneer Hi-Bred International, Inc.


Canada
Regulatory Approvals
Food
Submission to Health Canada
Molecular, nutritional, toxicology, allergenicity reviews. 60 day public comment under voluntary Notice of Submission.*
Questions
Request for additional information
No Questions
Food Rulings Committee
Hold for Feed, Environment Authorizations
Food Use Authorization
~24 months
Feed
Submission to Feed Section, Canadian Food Inspection Agency
Molecular, nutritional, toxicology and allergenicityreviews. 60-day public comment under voluntary Notice of Submission.*
Questions
Request for additional information
No Questions
Hold for Food, Environment Authorizations
Feed Use Authorization
~24 months
Environment
Confined Field Testing (Local)
Submission to Plant Biosafety Office, Canadian Food Inspection Agency
Molecular, environmental safety reviews. 60-day public comment under voluntary Notice of Submission.*
Questions
Request for additional information
No Questions
Hold for Food, Feed Authorizations
Environmental Release Authorization
~24 months
*Public comment period only happens once under this process.
Source: Pioneer Hi-Bred International, Inc.

European Union

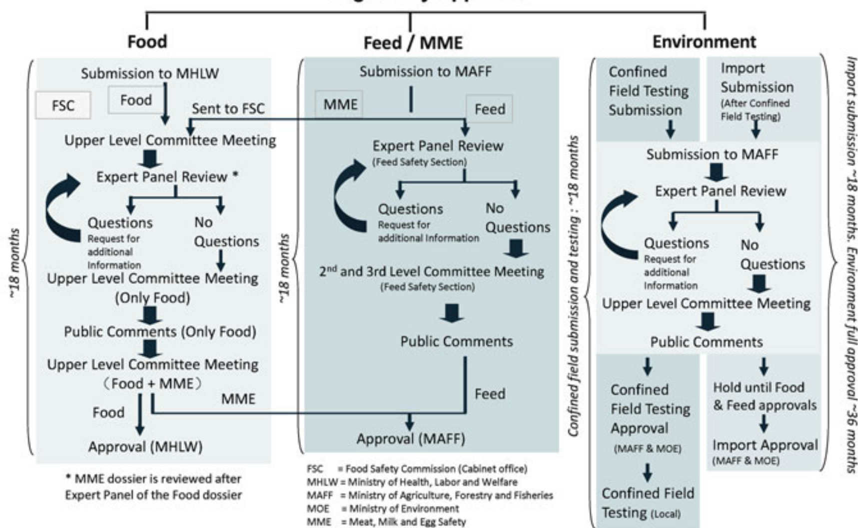
Food/Feed/Import or Cultivation [Regulation (EC) No 1829/2003]



Approximate Approval Period: *Food/Feed/Import: 30 months (EFSA Opinion) + 12 months (Commission Approval)*
Food/Feed/Cultivation: > 4 years

Source: Pioneer Hi-Bred International, Inc.

Japan



Source: Pioneer Hi-Bred International, Inc.

Chapter 17
Facilitating Market Access for GE Crops Developed Through Public Sector Research

Nina V. Fedoroff and Roger Beachy

Abstract Crops modified by recombinant DNA techniques have been an unqualified success from scientific, environmental, and economic perspectives. Generally known as GE (genetically engineered), GM (genetically modified), or GMOs (genetically modified organisms), such crops have been adopted by farmers at a historically unprecedented rate. The farm income benefit attributable to GE crops from 1996 to 2008 was US $52 billion, half in the developed and half in the developing world. The environmental and health impacts have all been positive, including substantial reductions in the use of pesticides and herbicides, as well as a significant reduction in mycotoxin contamination of maize. GE crops have caused neither environment damage nor either animal or human health problems. However, bringing a new GE variety to farmers is far more complex and costly than releasing a new variety created by older methodologies because unique regulatory requirements are imposed on crops modified by molecular methods, creating major barriers to their development and introduction. The lower profitability of fruit and vegetable than commodity crops discourages investment by seed companies, while universities and other public sector research organizations that have traditionally produced new varieties of such crops are excluded by both the financial and technical requirements of regulatory compliance.

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Keywords Crop domestication • FFDCA • FIFRA • PIPRA • Plant Quarantine Act • Plant Pest Act • Regulatory history • Risk assessment • Regulatory impact • Specialty Crop Regulatory Assistance • TSCA

17.1 Introduction

From scientific, environmental, and economic perspectives, crops modified by recombinant DNA techniques have been an unqualified success. Generally known as GE (genetically engineered), GM (genetically modified), or GMOs (genetically modified organisms),[1] such crops have been adopted by farmers at a historically unprecedented rate. Commencing with 1.7 million ha in 1996, the first year of commercial production, the global hectarage expanded to 148 million in 2010 (James 2011). From 1996 to 2008, the farm income benefit attributable to GE crops was US $52 billion, half in the developed and half in the developing world (Brookes and Barfoot 2010). The environmental and health impacts have all been positive, including substantial reductions in the use of pesticides and herbicides, as well as a significant reduction in mycotoxin contamination of maize (Brookes and Barfoot 2010; Wu 2006). None of the predictions that GE crops would damage the environment or cause either animal or human health problems have been realized.

And yet the number of different crops improved using molecular methods remains very small. The vast majority of hectarage planted to GE crops is devoted to cotton, soybeans, corn and canola (James 2011). All are commodity crops; three are major components of livestock feeds and the fourth is a fiber crop. The use of GE techniques to develop varieties that are protected against biotic and abiotic stresses in the crop environment and to improve crops that are primarily consumed as food by people, including many grains, beans, fruits and vegetables, is still rare. It is becoming increasing clear that the regulatory requirements imposed on GE crops create a major barrier to the development and introduction of such crops. Bringing a new GE variety to farmers is far more complex and costly than releasing a new variety created by older methodologies because the regulatory requirements are imposed solely on crops modified by molecular methods. Neither the large seed companies that have commercialized GE varieties of the commodity crops nor smaller start-up companies can readily afford to commercialize seeds of the much less profitable fruit and vegetable (horticultural) crops. Universities and other public sector research organizations that have traditionally produced new varieties of such crops are poorly prepared, both financially and technically, to commercialize GE crops, and few have been successful. Here we review how this situation developed, the current state of innovation, and possible paths to ameliorating the situation.

[1] Genetically modified (GM) is a much broader concept than the term 'genetically engineered (GE),' which we generally use herein to denote organisms modified by molecular techniques.

17.2 Historical Perspective on Crop Domestication and Improvement

Selecting and modifying plants to serve humans and their animals as sources of food, feed and other products, such as fiber, wood, medications and other specialty products, is deeply embedded in human cultures. Domestication of our major modern grains, including corn (maize), wheat, and rice, began many thousands of years ago (Doebley 2004; Dubcovsky and Dvorak 2007; Izawa et al. 2009). Common traits selected during domestication include attachment of seeds to the plant until ready for human harvesting (non-shattering rachis), reduction in the numbers of tissue layers that enclose seeds, and both more seeds and larger seeds. Analysis of specimens collected from archeological sites suggests that major steps in crop domestication occurred in the neighborhood of 10 millennia ago (Doebley 2004; Dubcovsky and Dvorak 2007; Izawa et al. 2009). Recent dramatic increases in crop productivity came from the application of insights from scientific advances in the understanding of plant nutrition, genetics, and breeding, as well as the invention of synthetic fertilizers and the mechanization of agriculture (Miller 2008; FAO Report 2000). The development of hybrid corn, and the identification of dwarfing mutations in wheat and rice, as well as the increased use of chemical and radiation mutagenesis combined with increasingly sophisticated breeding programs to produce a plethora of higher yielding and increasingly diverse grains, legumes, fruits and vegetables. Coupled with advances in mechanization, including improved tillage, planting and harvesting tools, crop productivity dramatically increased over the course of the twentieth century.

Early in the last century, much of the plant breeding in the U. S. was conducted in public institutions, such as land grant universities and the U. S. Department of Agriculture's (USDA) agricultural experiment stations, as part of the government's long-standing effort to bring science to farmers. Commercialization of agriculture, with historical roots in the seed and bulb trades of previous centuries, entered a new era with the development of hybrid corn and the founding of the Pioneer Hi-Bred Corn Company by Henry Wallace in 1926 to produce hybrid seed corn (Fedoroff and Brown 2004).

At the time, however, plant varieties were not viewed as human inventions and it was not until 1930, stimulated by the complaints of the brilliant and independent plant breeder Luther Burbank, that Congress accorded plant varieties their first legal status under the Plant Patent Act (PPA) of 1930 (Fedoroff and Brown 2004). Protection of plant varieties as intellectual property (IP) by the Plant Patent Act was confined to asexually propagated plants. Protection was later extended to sexually propagated plants by the Plant Variety Protection Act (PVPA) of 1970, which was further updated in 1994 (USDA 2005). The PVPA requires a new variety to be distinctive, uniform and genetically stable to qualify for IP protection. It is important to note that plant modification techniques now regarded as "traditional" include chemical and radiation mutagenesis, spontaneous mutagenesis in tissue culture, embryo rescue, interspecific crosses, and chemical polyploidization (Fedoroff and

Brown 2004). Varieties created by the application of such methods can be patented and their release is not further regulated by the U. S. government. While there have been a few cases of crops bred by traditional techniques for such qualities as insect resistance that proved to have deleterious effects on people, including the nausea-inducing Lenape potato and a rash-inducing celery variety, these stand as exceptions among the many thousands of new varieties released over the past century (Zitnak and Johnston 1970; Seligman et al. 1987). Thus the criteria of uniqueness, uniformity and stability, together with the prudence of the plant breeder, have sufficed for the safe introduction of new plant varieties as food and feed crops.

Basic discoveries in genetics and biochemistry through the first half of the twentieth century laid the groundwork for the genomic and biotechnology revolutions of the last half of the century (Fedoroff and Brown 2004). Upon its rediscovery at the turn of the twentieth century, the pioneering work of Gregor Mendel in the nineteenth century rapidly gave rise to the discipline of genetics. Geneticists first localized genes to chromosomes, structures that are located in cell nuclei. Researchers then showed that genes reside in the deoxyribonucleic acid (DNA) component of chromosomes. Mid-century was marked by the publication of Watson and Crick's elegant model of DNA structure, which immediately offered insights into the biochemical mechanisms underlying the inheritance of traits through genes (Watson and Crick 1953). The discovery of DNA-cleaving restriction enzymes, the identification of small chromosomes (plasmids) in bacteria, and the characterization of viruses made it possible to assemble the first "recombinant" DNA (rDNA) molecules, hybrids of a bacterial plasmid or a viral genome and a piece of DNA taken from a different organism. 'DNA cloning' is the process by which such an rDNA plasmid or virus is amplified in bacteria to produce a sufficient quantity to analyze by physical methods. The adoption of rDNA and cloning methods, in turn, set the stage for the invention of rapid DNA sequencing techniques and the genomic revolution. Development of methods to introduce DNA into plant cells, including particle bombardment and genetic transformation mediated by the soil bacterium *Agrobacterium tumefaciens*, made it possible to introduce new or altered genes into crop plants.

17.3 The History of GE Regulation

The first recombinant DNA constructs made with viral and plasmid DNAs aroused concerns in some of the most prominent molecular biologists of the era, several of whom authored a joint note in *Science* magazine in 1974 suggesting that certain types of experiments should not be done until the potential hazards could be evaluated (Berg et al. 1974). The note further requested that the National Institutes of Health (NIH) establish an advisory committee to (1) oversee an experimental program to evaluate the potential biological and ecological hazards, (2) develop containment procedures to minimize the spread of recombinant molecules, and (3) devise guidelines for scientists working with recombinant DNA (Berg et al. 1974). This publication was followed by a conference convened in Asilomar, California, to

make initial recommendations for working with recombinant DNA and organisms containing recombinant DNA. Many, but far from all, participants subscribed to the notion that regulation was necessary. Indeed, Nobel Laureate Joshua Lederberg predicted that the very process of regulating recombinant DNA would make people think it was dangerous, whether it was or not (Watson 2003).

A committee was duly constituted and named the NIH Recombinant DNA Advisory Committee, better known as the RAC. The RAC developed guidelines for the conduct of recombinant DNA research, the first version of which was highly restrictive and even prohibited the construction of certain kinds of rDNA molecules (NIH 1976). While the RAC was solely advisory to the Director of the NIH, then Dr. Donald Fredrickson, it acquired a stature and influence well beyond its official status (Fredrickson 2001). Investigators and companies alike brought their nascent rDNA projects and products to the RAC for approval. Although the guidelines were technically voluntary, both public and private sector researchers complied with them. There were a few exceptions and several careers were seriously damaged by publicized violations of the guidelines.

Despite the somewhat onerous containment requirements for working with recombinant DNA, the technology proved extremely powerful and caught on rapidly. Recombinant organisms of many different kinds and harboring different rDNA constructs were created within a few years, with no indications of unexpected deleterious properties. Experiments to determine whether or not rDNA molecules could escape from the common laboratory bacterium *E. coli* and be transferred to gut bacteria in animals and people yielded negative results. As a consequence, much of the early work of RAC addressed the strictures and operational requirements of the guidelines. As data accumulated, the RAC progressively approved the exemption of more and more categories of applications from further regulation. However, when the first requests to field-test plants produced using rDNA technology reached the RAC in roughly 1984, all of this began to change.

Representatives of the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (USDA, APHIS), the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) began to attend RAC meetings, initiating discussion about their roles in regulating plant applications of rDNA technology (Fedoroff and Brown 2004). The Office of Science and Technology Policy (OSTP) created the Biotechnology Science Coordinating Committee (BSCC) to bring together the various regulatory agencies to work out federal policy for regulating GE organisms. The BSCC produced a document titled "Coordinated Framework for the Regulation of Biotechnology" (OSTP 1986). The committee concluded that the use of rDNA techniques was not inherently risky and therefore did not require new regulatory legislation, but could be regulated under existing statues.

The three agencies identified as having regulatory authority were the USDA, the EPA and the FDA. As specified by the Coordinated Framework, each agency identified existing legislation under which it could regulate rDNA organisms. The USDA used the Plant Quarantine Act of 1912 and the Federal Plant Pest act of 1957, under which it has the power to decide if a new plant variety or microorganism is likely to become a pest. Since two of the organisms used in creating GE plants,

Agrobacterium tumefaciens and cauliflower mosaic virus (CaMV), the source of the commonly used CaMV 35S promoter, were both on the list of plant pests, APHIS was able to capture most emerging GE plant applications for case-by-case regulation as "regulated articles." The USDA further extended its definition of regulated articles to organisms that contain DNA from different genera, which extended coverage to essentially all GE plants. A developer using rDNA techniques must obtain a permit to carry out field tests and then eventually petition APHIS to "deregulate" the plant in order to be able to release a new variety of GE crop. The petition must describe the genes, regulatory sequences, and transformation procedure used, analyze the plant's genetic and agronomic traits, and provide data on the environmental consequences of growing the plant. This, in turn, triggers an environmental assessment by APHIS.

The EPA identified two existing statutes, the Toxic Substances Control Act (TSCA) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), under which it could regulate GE organisms (Fedoroff and Brown 2004). These statutes were created to allow the EPA to trace new industrial chemicals (TSCA) and to assess the toxicity of new chemicals and living organisms used to control fungi, insect and animal pests (FIFRA). To stretch these statues to cover GE organisms, the EPA defined them as "new," meaning developed through significant human intervention. In practice, only organisms modified by rDNA technology are considered "new." Using these two laws, the EPA regulates pesticidal properties of GE plants, such as corn expressing an insect-specific toxin gene from the bacterium *Bacillus thuringiensis* (Bt) and virus-resistant papaya expressing a fragment of the viral genome. The developer must provide the EPA with data on the inserted genes and their products, as well as potential risk and benefits to the environment in the form of an Environmental Risk Assessment. Furthermore, EPA can require the breeder to prepare a resistance management plan to reduce the likelihood that the target insect or disease becomes resistant to the protective agent produced by the GE crop plant. It also requires data on the toxicity of the protective agent to animals and humans and sets tolerance levels for residues allowed in food.

The FDA's regulatory authority derives from the 1938 Federal Food, Drug, and Cosmetic Act (FFDCA). The FDA directed developers of a GE variety to consult with the agency on safety and regulatory questions and recommended that the new variety be compared to a standard variety of the same crop to establish "substantial equivalence." The developer provides data comparing the composition of the new food with that of a standard product already in the marketplace and on the safety of the new component, generally either a protein or small molecule, expressed in the GE variety. The FDA analyzes the data submitted as part of the consultation process. A more stringent review process is required for foods that have been altered in nutrient composition.

Alarmed by the emerging regulatory environment for GE crops, the Council of the National Academy of Sciences issued a white paper in 1987 titled "Introduction of Recombinant DNA-Engineered Organisms into the Environment: Key Issues" (NAS 1987). It underscored the fact that there was no evidence that the use of rDNA techniques to modify organisms was inherently dangerous and that the genetic

engineering of organisms raised no new environmental risks per se. It concluded that regulation should be based on the product, not the process by which it was created. Paradoxically, although the intent of the Coordinated Framework was to avoid creating the perception of hazard where none was known to exist, the very process of using statutes created to regulate toxic substances, plant pests and food contaminants created precisely such a perception. Under the Coordinated Framework, the USDA regulated GE plants as potential agricultural pests, the EPA regulated them as pesticides (later renaming them "plant incorporated protectants" or PIPs), and the FDA regulated them as potential threats to food safety.

In Europe, organisms modified by rDNA techniques were viewed as a separate category from the outset and the regulatory processes were subsequently focused only on such organisms (OECD 1986, 1993). In 1990, the EU established a process for approving the "deliberate release" of GE organisms that required the approval of either all member states or a majority of a committee made up of representatives of member states (EC 1990). Despite the complexity of this process, the EU approved 18 GE crops for commercial marketing between 1992 and 1998 (Sheldon 2004). Regulatory approvals reached a political impasse in 1998 and in 1999, the EU Council (EC) formalized a moratorium on approvals to market GE crops until it could be demonstrated that there was neither a human health nor an environmental impact (the precautionary principle) and that they satisfied labeling and traceability requirements. Moreover, Denmark, France, Greece, Italy and Luxembourg declared they would block future GE crop approvals altogether, effectively imposing a total moratorium on GE crops (Sheldon 2004). The EC's approval in 2010 of commercialization of the genetically modified Amflora potato, developed by BASF for non-food industrial purposes, was the first GE crop approval since 1998 (EC press release 2010).

17.4 The Impact of the Regulatory Environment on Innovation

The number of approved GE traits remains small (primarily insect resistance and herbicide tolerance) and the major GE crops are either predominantly produced for feed (soybeans, corn, canola) or fiber (cotton). Traits that affect the quality of foods, such as color, taste, nutritional value, and freshness (extended shelf life), or remove toxic and allergenic components, are almost absent from the marketplace, with the exception of DuPont's recently approved high-oleic soybean (http://www.nytimes.com/gwire/2010/06/07/07greenwire-as-us-approves-gm-soybean-dupont-and-monsanto-80269.html). Even the long-in-development Golden Rice, enriched in the vitamin A precursor β-carotene, has yet to be released to farmers and consumers (Potrykus 2010). As Potrykus notes, getting a GE crop to market takes an order of magnitude more money and many more years than getting a conventional variety to market. The ringspot virus-resistant papaya remains virtually the only public-sector GE product in commercial production (Tripathi et al. 2007).

Analyses of the agricultural biotechnology products in development suggest that the EU's 1998 effective moratorium on GE approvals had a profound effect on the "innovation pipeline" (Graff et al. 2009). These authors note that product quality "…innovations introduced after 1998 had only a 0.6 % chance of reaching regulatory filing and 0.3 % chance of getting to market, relative to a 4.6 % chance of reaching regulatory filing and a 1.7% chance of getting to market for innovations introduced before 1998." At the same time, discoveries having the potential to impact product quality, particularly that of specialty crops such as fruits, vegetables, legumes and minor grains, have continued to increase as a consequence of investments in research (Graff et al. 2009; Miller and Bradford 2010). Miller and Bradford (2010) report that governmental regulatory bodies of 24 countries have approved or deregulated 84 unique plant and trait combinations since 1992. While about half as many specialty crop as commodity crop applications were approved between 1992 and 2002, only 5 % as many have been approved since 2003. Only 2 of the 21 approvals granted for specialty crops since 1992 were granted after 2000 (Miller and Bradford 2010).

While consumer rejection is frequently cited as a factor discouraging the development of GE specialty crops, the results of a number of surveys suggest that if GE foods offered health or taste advantages, consumers would buy them, even at premium prices (Rommens 2010). Thus it is by now unavoidably obvious that the major bottleneck in bringing quality-enhanced GE specialty crops to consumers lies in the regulatory process, its cost and complexity. Regulatory compliance costs for the introduction of insect-resistant maize and herbicide-tolerant maize, for example, have been estimated at between $6 million and $15 million dollars (Kalaitzandonakes et al. 2007). Such costs are simply out of proportion to the market value of most specialty crops. Furthermore, both the cost and complexity of complying with the regulations is beyond the capacities of either academic researchers or small start-up companies. Thus the regulatory requirements in place, even in the pro-GE U. S., essentially price out all developers except the large biotechnology companies and preclude the development of the very crops and products that have the possibility of increasing consumer acceptance, boosting the value of the horticultural crop industry and decreasing its ecological footprint through decreased use of chemicals to control pests and diseases.

17.5 The Way Forward

Given the impending effects of climate change and continued population growth, it is critically important to be able to make use of the substantial body of molecular and genetic knowledge of plants accumulated in recent decades. The anticipated increase in demand for food and the increasing demand for non-food and feed uses of agriculture, together with the anticipated negative impact of climate change, will put unparalleled pressure on agroecosystems. Plants and animals will need to be adapted to withstand the environmental stresses, diseases and pests anticipated in a

warming climate (Battisti and Naylor 2009). Molecular methods of breeding and crop improvement make it possible to transfer desirable genes from wild relatives while avoiding the addition of unwanted DNA or unwanted mutations by traditional breeding and mutagenesis techniques. These methods also make it possible to introduce traits that allow a crop to withstand biotic and abiotic stresses for which resistance genes have not been identified in a sexually compatible plant. And they make it possible to improve the nutritional value of widely used crops that have a high caloric value. This will necessitate making it easier for researchers to use rDNA techniques to improve a wide variety of crop plants and to get GE varieties to farmers.

Use of the full range of relevant technologies, including genetic engineering, can be facilitated in two ways: (1) helping developers, be they public or private sector, to comply with current regulatory requirements and (2) changing the regulatory requirements in the light of the more than three decades of accumulated experience with GE plants, including 15 years of successful commercialization of GE crops. An additional impediment that public sector researchers, especially those in universities, often encounter is that the molecular constructs they have long used in research are patent-protected and must be licensed at some cost before a GE plant can be considered for commercialization. This was one of the initial stumbling blocks faced by the academic developers of Golden Rice (Potrykus 2010).

Several organizations have been established in recent years to assist public-sector researchers and small businesses with GE crop development, introduction and commercialization. Commencing with a Policy Forum published in Science magazine in 2003, a group of university and public research sector administrators established the Public-Sector Intellectual Property Resource for Agriculture, widely known by its acronym PIPRA, supported by the Rockefeller and McKnight Foundations (Atkinson et al. 2003). PIPRA seeks to assist both public and private sector crop developers to assess the IP terrain, to develop licensing and other types of agreements, and to formulate a commercialization or product release strategy (PIPRA 2011). PIPRA has also established research laboratories at the University of California, Davis, that focus on agricultural biotechnology, performing laboratory research as well as greenhouse and field testing (PIPRA 2011). An Australian organization associated with the Queensland University of Technology, The Center for Application of Molecular Biology to International Agriculture (CAMBIA), similarly seeks to reduce the IP barriers for agricultural biotechnologies and provide "open source" molecular technology packages that can be used to develop GE plants unimpeded by patents (CAMBIA 2011).

The Specialty Crop Regulatory Assistance (SCRA) program grew out of a workshop on the impact of the regulatory process on public sector research in agricultural biotechnology sponsored by the Cooperative State Research Extension and Education System (CSREES; now National Institute of Food and Agriculture, NIFA), the Agricultural Research Service (ARS) and APHIS in 2004 (USDA Workshop 2004). The organizers of the workshop commissioned a recently published assessment of the impact of the regulatory process on commercialization of GE specialty crops (Miller and Bradford 2010). Because many developers of GE

specialty crops are somewhat naïve about the actual regulatory procedure, they may abandon a potentially useful product (A. McHughen 2011, personal communication). A workshop in December of 2011 conducted hands-on exercises with federal regulators to show clients exactly how the regulators approach a dossier for APHIS deregulation, FDA consultation and EPA approval of a potential product (SCRA Workshop 2011). The workshop included independent consultants with experience in handling dossiers for clients, to provide an opportunity for small market and specialty GM crop developers to interact both with the regulators who will assess a dossier and consultants who might be able to help navigate the regulatory system. The intent of the workshop was to spur developers to move their products forward and encourage others to continue their research and development efforts by showing that it is indeed possible to get a product to market (A. McHughen 2011, personal communication).

While it is important for developers to be familiar with the process of meeting existing regulatory requirements, it is also critically important to revisit the regulations themselves in the light of scientific knowledge that has accumulated since GE crops were first developed and commercialized. Twenty-five years after its publication, the 1986 Coordinated Framework for the Regulation of Biotechnology continues to be the guidance for the federal regulation of rDNA organisms. Had the remarkable capacity of the RAC to respond to the growing safety record of recombinant DNA research by relaxing the guidelines persisted through the era of plant applications, we would have a very different research and development environment for GE crops today.

A 2004 NRC study reaffirmed that there was no scientific justification for singling out rDNA techniques as more risky than other plant genetic modification techniques now regarded as conventional, including tissue culture, chemical and radiation mutagenesis, wide crosses, embryo rescue and polyploidization (NRC 2004). In 2010, the European Commission published a summary of the past decade of EC-sponsored research in the European Union on the safety of genetically engineered organisms (EC 2010), which followed on its 2001 document summarizing the first 15 years of such research (EC 2001). It states: "The main conclusion to be drawn from the efforts of more than 130 research projects, covering a period of more than 25 years of research and involving more than 500 independent research groups, is that biotechnology, and in particular GMOs, are not *per se* more risky than e.g. conventional plant breeding technologies." The European Union has spent more than EUR 300 million on GE biosafety research since 1982 (EC 2010).

To begin reforming the regulatory process, it is critical to recognize and acknowledge that the current approach is *de facto* process-based, not product-based, as it was intended to be and as mandated (OSTP 1992). Thus, for example, APHIS categorizes plants into which *Agrobacterium* Ti plasmid sequences and plant viral DNA sequences, such as the CaMV 35S promoter have been introduced by rDNA methods as "regulated articles" because the process used to introduce a gene involves a sequence derived from a plant pathogen or introduces a sequence derived from a plant pathogen. Based on the accumulated experience of more that 25 years and many hundreds of studies, there is simply no rationale for categorizing plants that

contain well-characterized fragments of T DNA or viral DNA as plant pests, as such plants have not succumbed to the diseases associated with *Agrobacterium* or virus fragments that are used solely to regulate expression of transgenes. Furthermore, many plant genomes harbor viral sequences without consequence, and expressing a small fragment of a viral genome can protect a plant from infection, seriously upending the rationale behind the categorization (Tripathi et al. 2007). Thus an essential first step in redefining regulation is to revise the blanket designation of a plant as a plant pest based on the parent organism and re-focus the regulatory process on the trait that has been introduced (Bradford et al. 2005). However, this alone should not be viewed as a panacea, as it is possible, judging by the Canadian experience, to create a product-based regulatory process that is nonetheless unjustifiably complex and onerous (Smyth and McHughen 2008).

The next essential element of regulatory reform is to identify types of genetic modifications, as well as individual genes, that can be exempted from further regulation based upon a history of safe use. For example, the safe history of use of *B.t.* toxin genes and certain herbicide tolerance genes argues for reduced oversight. Given the explosion of knowledge about regulatory mechanisms mediated by small RNAs, we now know that gene suppression methods based on small RNAs have the same kinds of effects on gene expression as conventional mutations and need not be regulated *per se* (Frizzi and Huang 2010). A number of individual genes coding for non-toxic proteins, marker genes such as β-glucuronidase (GUS) and the green fluorescent protein (GFP), and even some antibiotic resistance markers that have achieved "generally regarded as safe" or GRAS classification, are clear candidates for exemption (Bradford et al. 2005). It should be noted that several such protein, including GUS but not GFP, have already been granted exemptions from the requirement of a tolerance under FFDCA when used as inert ingredients in PIPs, which means that they do not require further review.

One of the requirements of the current regulatory approach that can be eliminated in the light of current knowledge is the requirement for separate approval of each independent transformation event. When the original regulations were formulated, no plant genomes had been mapped or sequenced. Today we know infinitely more about plant genomes and recognize their inherent lability, as well as the abundance of non-coding DNA in genomes. Early fears that some insertions would cause unanticipated deleterious effects that would remain undiscovered until crops were marketed for some years have simply not been borne out by the results of a quarter century of biosafety research on GE plants (EC 2010) and 15 years of commercial growth of GE crops (James 2011). Hence there is no justification for in-depth characterization of insertion sites and the independent safety assessment of each insertion event. Indeed, there is a far more compelling rationale for abolishing the requirement because it complicates the transfer of traits to locally adapted varieties, most efficiently done by transformation, not by genetic crosses (Bradford et al. 2005).

The next essential task is to develop a regulatory framework based on current best assessments of the actual hazard posed by the modified organism, as originally proposed in an NRC report defining a decision framework for GM organisms (NRC 1989; Bradford et al. 2005). Thus, for example, the addition of a gene encoding a

non-toxic, non-allergenic protein to a well-characterized crop plant that has no immediate weedy relatives has a sufficiently low probability of causing a health or environmental problem so that adequate information can be collected in the course of the characterization required for patenting of the new variety. GE organisms expressing proteins known to have physiological effects on animals or humans or which are designed to alter their behavior or reproduction, by contrast, require both more careful assessment and, in some cases, more effective containment measures than necessary for well-characterized crop plants. The adoption of a modernized framework can support the exemption from regulation of some classes of transgenes and crops that contain them. Reducing the cost of de-regulation will stimulate invention and innovations flowing from the public sector, as well as from biotechnology companies.

Finally, there is at least one new methodology in development and testing that results in the highly specific modification of plant genes without creating a transgenic plant. This technology is based on synthetic proteins that fuse a well-characterized synthetic sequence-specific DNA-binding protein to a nuclease domain that cleaves double-stranded DNA (Shukla et al. 2009; Mahfouz et al. 2011). Transient expression of the gene encoding the nuclease, together with the introduction of a DNA sequence bearing homology to the site targeted at which the DNA is cleaved, permits a variety of highly specific DNA changes to be introduced, ranging from single nucleotide changes to deletions and insertions of DNA sequences of various sizes. Because untargeted insertion of DNA sequences happens at a much lower frequency than the targeted modifications, regenerated plants containing only the intended DNA sequence change can be identified readily. These methods, and perhaps others, will permit the production of plants with highly specific genetic changes, but containing no recombinant DNA. This makes the resulting plants much more like those resulting from spontaneous mutation or mutations induced by cell culture, chemicals or radiation, except that the changes are far more precise, altering only the intended target sequences in the genome. Such plants should not be regulated as transgenics.

17.6 Conclusion

GE crops have been developed and commercialized both safely and very successfully over the past three decades. Today, GE crops are grown in 29 countries by more than 15 million farmers, 90 % of whom are resource-poor, small-holder farmers (James 2011). However, the commercial GE market is dominated by just four different crops (cotton, corn, canola, and soybean) and two traits (insect-resistance and herbicide tolerance), both of which are valuable to farmers, but of little interest to consumers. The number of GE specialty crops and the variety of GE traits in the commercial pipeline that are of interest to consumers, such as nutritional value, shelf-life, appearance or taste, remains very small. Several studies have documented the constriction of what should be an expanding innovation pipeline and identified

the high cost and complexity of the regulatory requirements as the major factor inhibiting the introduction of such crop-trait combinations from both public and private sector research sectors. We conclude that there is a critical need both to assist developers to comply with existing regulations and to revise the regulatory framework in the light of results of 25 years of biosafety research and 15 years of large-scale commercial GE crop production.

References

Atkinson RC, Beachy RN, Conway G, Cordova FA, Fox MA, Holbrook KA, Klessig DF, McCormick RL, McPherson PM, Rawlings HR 3rd, Rapson R, Vanderhoef LN, Wiley JD, and Young CE (2003) Intellectual property rights. Public sector collaboration for agricultural IP management. Science 301:174–175

Battisti DS, Naylor RL (2009) Historical warnings of future food insecurity with unprecedented seasonal heat. Science 323:240–244

Berg P, Baltimore D, Boyer HW, Cohen SN, Davis RW, Hogness DS, Nathans D, Roblin R, Watson JD, Weissman S, Zinder ND (1974) Biohazards of recombinant DNA. Science 185:303

Bradford KJ, Deynze AV, Gutterson N, Parrott W, Strauss SH (2005) Regulating transgenic crops sensibly: lessons from plant breeding, biotechnology and genomics. Nat Biotechnol 23:439–444

Brookes G, Barfoot P (2010) GM crops: global socio-economic and environmental impacts 1996–2008. PG Economics Ltd, UK. http://www.pgeconomics.co.uk/. Access date 25 Apr 2011

CAMBIA (2011) http://www.cambia.org/daisy/cambia/home.html. Access date 25 Apr 2011

Doebley J (2004) The genetics of maize evolution. Annu Rev Genet 38:37–59

Dubcovsky J, Dvorak J (2007) Genome plasticity a key factor in the success of polyploid wheat under domestication. Science 316:1862–1865

EC (1990) Council directive 90/220/EEC of 23 April 1990 on the deliberate release into the environment of genetically modified organisms. http://eur-lex.europa.eu/smartapi/cgi/sga_doc?smartapi!celexapi!prod!CELEXnumdoc&lg=EN&numdoc=31990L0220&model=guichett

EC (2001) EC-sponsored research on safety of genetically modified organisms. In: Kessler C, Economidis I (eds) http://ec.europa.eu/research/quality-of-life/gmo. Access date 25 Apr 2011

EC (2010) A decade of EU-funded GMO research (2001–2010). In: Economidis I, Cichocka D, Högel J (eds) http://ec.europa.eu/research/biosociety/pdf/a_decade_of_eu-funded_gmo_research.pdf. Access date 25 Apr 2011

EC press release (2010) http://europa.eu/rapid/pressReleasesAction.do?reference=IP/10/222. Access date 25 Apr 2011

FAO Report: The state of food and agriculture (2000) http://www.fao.org/docrep/x4400e/x4400e10.htm. Access date 25 Apr 2011

Fedoroff N, Brown NM (2004) Mendel in the kitchen: a scientist's view of genetically modified foods. Joseph Henry Press, Washington, DC

Fredrickson DS (2001) The recombinant DNA controversy: a memoir. Am Soc Microbiol, Washington, DC

Frizzi A, Huang S (2010) Tapping RNA silencing pathways for plant biotechnology. Plant Biotechnol J 8:655–677

Graff GD, Zilberman D, Bennett AB (2009) The contraction of agbiotech product quality innovation. Nat Biotechnol 27:702–704

Izawa T, Konishi S, Shomura A, Yano M (2009) DNA changes tell us about rice domestication. Cur Opin Plant Biol 12:185–192

James C (2011) Global status of commercialized biotech/GM crops: 2010. ISAAA brief 42. http://www.isaaa.org/

Kalaitzandonakes N, Alston JM, Bradford KJ (2007) Compliance costs for regulatory approval of new biotech crops. Nat Biotechnol 25:509–511
Mahfouz MM, Li L, Shamimuzzaman M, Wibowo A, Fang X, Zhu J-K (2011) De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. Proc Natl Acad Sci USA 108:2623–2628
Miller FP (2008) After 10,000 years of agriculture, whither agronomy? Agron J 100:22–34
Miller JK, Bradford KJ (2010) The regulatory bottleneck for biotech specialty crops. Nat Biotechnol 28:1012–1014
NAS (1987) Introduction of recombinant DNA-engineered organisms into the environment: key issues. National Academies Press, Washington, DC
NIH (1976) Recombinant DNA research. Guidelines. Fed Regist 41:27 902, 27 911–27 943
NRC (1989) Field testing genetically modified organisms: framework for decisions. National Academies Press, Washington, DC
NRC (2004) Safety of genetically engineered foods. National Academies Press, Washington, DC
OECD (1986) Recombinant DNA safety considerations: safety considerations for industrial, agricultural and environmental applications of organisms derived by recombinant DNA techniques. OECD, Paris
OECD (1993) Safety evaluation of foods derived by modern biotechnology—concepts and principles. OECD, Paris
OSTP (1986) Coordinated framework for regulation of biotechnology: announcement of policy and notice for public comment. Federal Reg 51:23, 302–323, 393
OSTP (1992) Exercise of federal oversight within scope of statutory authority: planned introductions of biotechnology products into the environment. Fed Regist 57:6753–6762
PIPRA (2011) http://www.pipra.org/. Access date 25 Apr 2011
Potrykus I (2010). Regulation must be revolutionized. Nature 466:561
Rommens CM (2010) Barriers and paths to market for genetically engineered crops. Plant Biotechnol J 8:101–111
Seligman PJ, Mathias CGT, O'Malley MA, Beier RC, Fehrs LJ, Serrill WS, Halperin WE (1987) Phytophotodermatitis from celery among grocery store workers. Arch Dermatol 123:1478–1482
Sheldon I (2004) Europe's regulation of agricultural biotechnology: precaution or trade distortion? J Agric Food Ind Org. doi:10.2202/1542-0485.1060
Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, Mitchell JC, Arnold NL, Gopalan S, Meng X, Choi VM, Rock JM, Wu Y-Y, Katibah GE, Zhifang G, McCaskill D, Simpson MA, Blakeslee B, Greenwalt SA, Butler HJ, Hinkley SJ, Zhang L, Rebar EJ, Gregory PD, Urnov FD (2009) Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature 459:437–441
Smyth S, McHughen A (2008) Regulating innovative crop technologies in Canada: the case of regulating genetically modified crops. Plant Biotechnol J 6:213–225
Tripathi S, Suzuki J, Gonsalves D (2007) Development of genetically engineered resistant papaya for papaya ringspot virus in a timely manner: a comprehensive and successful approach. Methods Mol Biol 354:197–240
USDA (2005) Plant Variety Protection Act. http://www.wipo.int/wipolex/en/details.jsp?id=5426. Access date 25 Apr 2011
USDA Workshop (2004) http://www.csrees.usda.gov/nea/biotech/pdfs/small_mkt.pdf. Access date: 25 Apr 2011
USDA Workshop (2011) http://specialtycropassistance.org/content.cfm?ID=241. Access date 25 Apr 2011
Watson JD (2003) A conversation with Jim Watson. http://calteches.library.caltech.edu/4081/1/Watson.pdf
Watson JD, Crick FHC (1953) A structure for deoxyribose nucleic acid. Nature 171:737–738
Wu F (2006) Mycotoxin reduction in Bt corn: potential economic, health, and regulatory impacts. Transgenic Res 15:277–289
Zitnak A, Johnston GR (1970) Glycoalkaloid content of B5141-6 potatoes. Am Potato J 47:256–260

Index

A

Adventitious presence, 128, 129, 136, 366–367
Alcaligenes xylosoxidans var. *denitrificans* (Axd), 106, 111–119, 121
Algae, 65, 75, 82, 90, 91, 98, 144
Animal Plant Health Inspection Service (APHIS), 3, 5–10, 12, 43, 45–48, 54, 61, 62, 64, 76, 77, 99, 106, 111, 115, 116, 122, 136, 276–278, 290, 294, 296, 346, 383, 387, 388
Animals, 2, 18, 40, 64, 98, 106, 142, 172, 276, 286, 305, 337, 380

B

Bacillus thuringiensis, 2, 41, 44, 52–55, 61, 62, 68, 69, 71, 86, 87, 100, 101, 131, 133, 134, 169, 180–254, 326, 351, 384
Bacteria, 5, 12, 41, 42, 61, 62, 65, 70, 75, 77, 78, 81, 83, 92, 98–100, 106–112, 115, 120, 121, 144, 298, 309, 311, 312, 316, 349, 382, 383
Baculovirus, 63, 64
Biocontrol, 71, 75, 100, 289
Biofertilizer, 8, 79, 82, 89
Biofuel, 8, 54, 79, 82, 85, 90, 91, 102
Biopesticide, 3, 64, 65, 72, 73, 75, 100, 106, 116, 279, 281
Biosafety, 12, 18, 21, 22, 28, 45, 76, 106, 116, 135, 144, 146, 157, 162, 292, 343, 344, 348, 358, 388–390
Biotechnology, 1, 18, 38, 60, 106, 136, 141, 151, 169, 236, 290, 313, 321, 327, 381
Bt resistance, 181, 182, 200, 202, 206, 212, 215–217, 220, 221
Bucket theory, 323, 324

C

Canada, 17–35, 37–56, 98, 99, 129, 133–136, 141–149, 151–165, 184, 275, 281, 295, 296, 311, 313, 337, 338, 342–347, 351–353, 355, 371, 373, 374
Canadian Environmental Protection Act (CEPA), 30–32, 141–149, 153, 154
Coexistence, 136
Commercial products, 23, 29, 35, 40, 90, 91, 99, 163, 274
Competitive displacement, 107
Contamination, 73, 90, 101, 128, 129, 136, 207, 380
Corn, 2, 62, 74, 100, 151, 159, 162, 171, 180–185, 191, 192, 198, 200, 205–208, 210, 211, 213, 214, 217, 220, 221, 224–227, 229–249, 251–254, 275, 330, 339, 340, 342, 347, 361
Cotton, 2, 28, 40, 107, 135, 171, 181, 274, 290, 330, 361, 380
Crop domestication, 380–382
Cross-pollination, 37, 51, 128, 152, 325, 356, 357
Cry toxins, 68, 174, 195, 196, 199

E

Ecological risk assessment, 173–175, 332
Environmental Impact Statement (EIS), 47, 48, 296–297, 316

C.A. Wozniak and A. McHughen (eds.), *Regulation of Agricultural Biotechnology: The United States and Canada*, DOI 10.1007/978-94-007-2156-2, Springer Science+Business Media Dordrecht 2012

Environmental Protection Agency (EPA), 2–11, 42, 43, 52–55, 60, 62–68, 72, 73, 75–86, 88, 90, 98–101, 116, 117, 167–176, 179–254, 277–281, 294, 354, 361, 383
Environmental Release, 2, 3, 5, 9, 28, 42, 43, 61, 67, 68, 72, 76, 81, 82, 130, 131, 145, 148, 151–165, 315
Experimental Use Permits (EUPs), 9, 10, 62, 65, 67–72, 168, 175
Exports, 145, 282, 338–343, 353, 356, 363, 369, 373, 374

F

FDA. *See* Food and Drug Administration (FDA)
Federal Food, Drug, and Cosmetic Act (FFDCA), 6–11, 49, 52, 59–92, 168, 169, 171, 176, 294, 314, 384, 389
Federal Insecticide, 8, 52, 69, 84, 181, 183, 185, 294
Ferality,
FFDCA. *See* Federal Food, Drug, and Cosmetic Act (FFDCA)
Field testing, 8, 9, 22, 25, 45, 61, 63, 67, 72, 81, 82, 92, 152, 158, 175–176, 277, 278, 329, 371
FIFRA. *See* Fungicide and Rodenticide Act (FIFRA)
Food and Drug Administration (FDA), 3–12, 42, 43, 49–51, 53, 55, 61, 64, 74, 90, 171, 277–279, 294, 309, 310, 314–316, 345, 351, 354, 383, 384, 387
Food Quality Protection Act (FQPA),
Fungi, 2, 65, 75, 81, 89, 98, 99, 144, 180, 383
Fungicide and Rodenticide Act (FIFRA), 3, 6–10, 52, 54, 59–92, 168, 169, 171, 175, 176, 181, 183, 185, 186, 188, 192, 222, 224, 237, 249, 294, 383

G

Gene silencing, 277
Genetically engineered (GE) crops, 2, 6, 9, 11, 78, 127, 128, 274, 278, 293, 294, 379–390
Genetically engineered (GE) insects, 285–298
Genetically engineered (GE) microorganism, 9, 59–92, 98
Genetically engineered organisms (GEOs), 3, 5–8, 40, 60, 66, 68, 77, 98, 293
Genetically engineered (GE) regulations, 59–92, 305–316, 382–385
Genetically modified organisms (GMOs), 38, 40, 42, 43, 49, 56, 61, 98–103, 142, 328, 379, 388
Genetic engineering (GE), 2–10, 12, 18, 38, 76–77, 98, 155, 276, 282, 287–289, 310–312
Globalization, 337–376

H

Homalodisca vitripennis, 108
Hybridization, 3, 127, 128, 171, 325
Hypothesis testing, 323–324

I

Imports, 7, 28, 32, 41, 43, 79, 142, 144–149, 152, 164, 292, 295, 339, 343, 347, 352, 353, 356, 364, 365, 367, 370, 371, 373, 376
Insect resistance management (IRM), 53, 55, 175, 179–254, 326, 327, 331–333, 361–362

L

Lentivirus, 308
Low level presence (LLP), 136, 137, 164, 165, 366–367

M

Microbial pest control agents (MPCA), 3, 9, 65–74, 77, 78, 84, 92
Micro-organisms, 5, 7–9, 32, 40, 59–92, 97–103, 118, 142–147

N

Novel traits, 18–20, 22, 23, 26–29, 44, 46, 128, 143, 151–165

P

Pest control, 3, 9, 18, 65–73, 76–78, 84, 98, 142, 146, 147, 153, 167, 182, 205, 237, 249, 288, 294, 296, 297, 322
Pesticide, 3, 5–11, 42, 43, 52, 54, 55, 61, 62, 65–67, 72–76, 78, 84, 86, 87, 90, 117, 146, 168, 169, 174, 175, 179–186, 200, 202, 207, 219–221, 244, 249, 254, 279, 280, 294

Pierce's disease, 107–113
Piggybac, 286, 308
Plant-incorporated protectants (PIPs), 3, 7–11, 52–54, 97, 101, 167–176, 179–254, 384, 389
Plant Pest Act, 43, 61, 75, 76
Plant pests, 6–8, 28, 43–45, 52, 54, 61, 75, 76, 99, 100, 128, 156, 161, 294–297
Plant Protection Act, 6, 7, 43, 64, 76, 90, 293, 294
Plant Quarantine Act, 13
Plants with Novel Traits (PNTs), 18, 25–27, 143, 151–165
Plenish™, 366, 370
Plum, 53, 101, 171, 231–282
Plum Pox Virus (PPV), 101, 231, 273–282
Policy, 2–5, 7, 11, 42, 44, 46, 49, 50, 54, 60, 64, 136, 153, 155, 159, 162, 229, 293, 296, 298, 324, 365–366, 369, 372, 373, 383, 387
Problem selection, 327, 328
Prunus, 275, 276, 282
Public-Sector Intellectual Property Resource for Agriculture (PIPRA), 282, 387

R
Refuge strategy, 187–189, 195, 201, 205, 209, 216, 217, 221–223, 236, 253, 254, 326
Regulated articles, 7, 8, 44, 48, 157, 175, 383, 388
Regulation, 1–13, 17–35, 37–56, 59–92, 98, 99, 102, 116–118, 141–149, 152–154, 156–163, 167, 181, 183, 185, 186, 254, 273–282, 292–295, 298, 305–316, 324, 332–334, 337–339, 342–346, 351, 354–356, 359, 363, 367, 373, 374, 382–384, 386–390
Regulatory compliance, 48, 56, 337–374
Regulatory history, 5
Regulatory impact, 385–386
Regulatory science, 321–334
Risk assessment, 5, 10, 26, 30, 32, 42, 43, 46, 55, 60, 64–75, 78–92, 101, 134, 142, 144–149, 152, 165, 168–176, 185, 186, 201, 227, 277, 278, 292–294, 298, 314, 322–330, 332–334
Rosaceae,

S
Seed spillage, 129, 136
Sharka, 275, 276
Simulation modeling, 179–254
Specialty Crop Regulatory Assistance (SCRA), 282, 387
Stewardship, 156, 162, 163, 186, 236, 244, 332, 337–374
Symbiotic control, 105–122

T
Toxic, 8, 32, 33, 39, 50, 52, 54, 60, 63, 74, 78, 79, 84, 102, 116, 117, 142, 145, 147–149, 154, 171, 173, 191, 199, 204, 205, 315, 316, 328, 389
Toxic Substances Control Act (TSCA), 8, 59–92, 106, 116, 117, 383
Trade, 20, 21, 23–25, 44, 137, 164, 180, 220, 243, 274, 289, 313, 338, 343, 345, 355, 360, 361, 363–369, 371, 372, 374, 381
Transfection, 306
Transgene flow,
Transgenic 7, 18, 38, 61, 116, 127, 169, 180, 273, 286, 306, 322, 349, 389
Transposon, 144, 286, 287, 306, 308
TSCA. *See* Toxic Substances Control Act (TSCA)

U
U.S. Coordinated Framework, 1, 4, 6, 8, 12, 40, 41, 50, 51, 53, 57, 58, 59, 73, 289, 344, 381, 383, 386

V
Vaccines, 99, 101–103, 153
Virus resistance, 52, 169, 276

X
Xylella fastidiosa, 88, 106, 108–110, 116, 117, 120, 121