

Assessment of the food safety issues related to genetically modified foods

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Summary

International consensus has been reached on the principles regarding evaluation of the food safety of genetically modified plants. The concept of substantial equivalence has been developed as part of a safety evaluation framework, based on the idea that existing foods can serve as a basis for comparing the properties of genetically modified foods with the appropriate counterpart. Application of the concept is not a safety assessment *per se*, but helps to identify similarities and differences between the existing food and the new product, which are then subject to further toxicological investigation. Substantial equivalence is a starting point in the safety evaluation, rather than an endpoint of the assessment. Consensus on practical application of the principle should be further elaborated. Experiences with the safety testing of newly inserted proteins and of whole genetically modified foods are reviewed, and limitations of current test methodologies are discussed. The development and validation of new profiling methods such as DNA microarray technology, proteomics, and metabolomics for the identification and characterization of unintended effects, which may occur as a result of the genetic modification, is recommended. The assessment of the allergenicity of newly inserted proteins and of marker genes is discussed. An issue that will gain importance in the near future is that of post-marketing surveillance of the foods derived from genetically modified crops. It is concluded, among others that, that application of the principle of substantial equivalence has proven adequate, and that no alternative adequate safety assessment strategies are available.

Keywords: biotechnology, genetic modification, genetic engineering, food crops, food safety, toxicology, substantial equivalence, legislation, risk assessment, profiling techniques, post market surveillance

Safety evaluation strategies

At an early stage in the introduction of recombinant-DNA technology in modern plant breeding and biotechnological food production systems, efforts began to define internationally harmonized evaluation strategies for the safety of foods derived from genetically modified organisms (GMOs). Two years after the first successful transformation experiment in plants (tobacco) in 1988, the International Food Biotechnology Council (IFBC) published the first report on the issue of safety assessment of these new varieties (IFBC, 1990). The comparative approach described in this report has laid the basis for later safety evaluation strategies. Other organizations, such as the

Organisation for Economic Cooperation and Development (OECD), the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) and the International Life Sciences Institute (ILSI) have developed further guidelines for safety assessment which have obtained broad international consensus among experts on food safety evaluation.

Organisation for Economic Cooperation and Development

In 1993 the OECD formulated the concept of substantial equivalence as a guiding tool for the assessment of

Concept of substantial equivalence

- * Starting point for safety assessment
- * Comparison between the GM organism and its closest traditional counterpart
- * Identification of intended and unintended differences on which further safety assessment should be focused

Figure 1. The concept of substantial equivalence.

genetically modified foods, which has been further elaborated in the following years (OECD, 1993a; OECD, 1996; OECD, 1998; Figure 1). The concept of substantial equivalence is part of a safety evaluation framework based on the idea that existing foods can serve as a basis for comparing the properties of a genetically modified food with the appropriate counterpart. The existing food supply is considered to be safe, as experienced by a long history of use, although it is recognized that foods may contain many anti-nutrients and toxicants which, at certain levels of consumption, may induce deleterious effects in humans and animals. Application of the concept is not a safety assessment *per se*, but helps to identify similarities and potential differences between the existing food and the new product, which is then subject to further toxicological investigation. Three scenarios are envisioned in which the genetically modified plant or food would be (i) substantially equivalent; (ii) substantially equivalent except for the inserted trait; or (iii) not equivalent at all. A compositional analysis of key components, including key nutrients and natural toxicants, is the basis of assessment of substantial equivalence, in addition to phenotypic and agronomic characteristics of the genetically modified plant.

In the first scenario, no further specific testing is required as the product has been characterized as substantially equivalent to a traditional counterpart whose consumption is considered to be safe, for example, starch from potato. In the second scenario, substantial equivalence would apply except for the inserted trait, and so the focus of the safety testing is on this trait, for example, an insecticidal protein of genetically modified tomato. Safety tests include specific toxicity testing according to the nature and function of the newly expressed protein; potential occurrence of unintended effects; potential for gene transfer from genetically modified foods to human/animal gut flora; the potential allergenicity of the newly inserted traits; and the role of the new food in the diet

Safety issues of GM foods

- (i) Genetic modification process
- (ii) Safety of new proteins
- (iii) Occurrence and implications of unintended effects
- (iv) Gene transfer to gut microflora
- (v) Allergenicity of new proteins
- (vi) Role of the new food in the diet
- (vii) Influence of food processing

Figure 2. Safety issues with regard to genetically modified foods.

(Figure 2). In the third scenario, the novel crop or food would be not substantially equivalent with a traditional counterpart, and a case-by-case assessment of the new food must be carried out according to the characteristics of the new product.

Key components of a specific crop for comparison with a genetically modified crop are described by Consensus Documents compiled by the OECD's Task Force for the Safety of Novel Foods and Feeds (OECD, 2001a). These documents provide useful guidance on which components should be minimally analysed.

International Life Sciences Institute

A consensus document has been prepared by ILSI Europe on evaluation of the safety of novel foods (Jonas *et al.*, 1996). This document provides background for data requirements for all novel foods, including foods and food ingredients derived from GMOs. For genetically modified foods this will include data on transgenic DNA; phenotype; and composition including gross composition, nutrients, anti-nutrients, and toxins. Substantial equivalence of the novel food to an appropriate counterpart can then be determined. There is a degree of freedom in choosing the level at which this comparison should be carried out, such as the food source, food product, and molecular levels. Similar to the OECD's and FAO/WHO's consensus views, the ILSI document defines three scenarios in which the novel food or food ingredient is characterized as (i) substantially equivalent to a reference food/ingredient; (ii) sufficiently similar; or (iii) not sufficiently similar. For novel foods and novel food ingredients that are not substantially equivalent, nutritional and toxicological data, and data concerning allergenic potential, need to be considered. A decision tree for testing genetically modified foods for allergenicity has been developed by ILSI in collaboration with the IFBC (Metcalf *et al.*, 1996). Three scenarios are considered where the source of the transgene may be: (i) a commonly

allergenic food; (ii) a less commonly allergenic food or other known food source; or (iii) without a history of allergenicity. Criteria used in the decision tree include:

- source of the transferred material;
- sequence homology of the transgene product to allergenic proteins;
- immunoreactivity of the transgene product tested with sera from individuals who are allergic to the source;
- stability of the transgene product under gastro-intestinal conditions, or under heat or other processing conditions.

IgE-binding tests are recommended with the new protein derived from known allergenic sources, using sera from individuals allergic to those sources, followed if necessary by skin-prick testing and double-blind food challenges. The decision-tree approach for new proteins derived from sources with no known history of allergenicity relies primarily upon sequence homology comparisons with known allergens, and on the stability of the protein under gastro-intestinal and food-processing conditions.

Food and Agriculture Organization/World Health Organization

FAO and WHO have been organizing workshops and consultations on the safety of GMOs since 1990. At the Joint FAO/WHO Consultation in 1996 (FAO/WHO, 1996) it was recommended that the safety evaluation should be based on the concept of substantial equivalence, which is 'a dynamic, analytical exercise in the assessment of the safety of a new food relative to an existing food'. The following parameters should be considered to determine the substantial equivalence of a genetically modified plant: molecular characterization; phenotypic characteristics; key nutrients; toxicants; and allergens.

The distinction between three levels of substantial equivalence (complete, partial, non-) of the novel food to its counterpart, and the subsequent decisions for further testing based upon substantial equivalence, are similar to those defined by OECD (1996).

The Codex Alimentarius Commission of FAO/WHO is committed to the international harmonization of food standards. Food standards developed by Codex Alimentarius should be adopted by the participating national governments. The Codex *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology has the task to develop standards, guidelines and other recommendations for genetically modified foods. During its first session in Chiba (Japan) in March 2000 (FAO/WHO, 2000a), definitions were agreed concerning the 'risk assessment' and 'risk analysis' of genetically modified foods. Risk assessment covers issues such as food safety, substantial equivalence and long-term health effects, while

risk analysis may include decision-making and post-market monitoring.

An Expert Consultation held in Geneva, Switzerland in May/June 2000 evaluated experiences gathered since the 1996 Consultation. Topics considered included substantial equivalence, unintended effects of genetic modification, food safety, nutritional effects, antibiotic resistance marker genes, and allergenicity. The Consultation endorsed the concept of substantial equivalence as a pragmatic approach for the safety assessment of genetically modified foods, and concluded that at present no suitable alternative strategies are available. Application of the concept is a starting point for safety assessment, rather than an endpoint. It identifies similarities and possible differences between the genetically modified food and its appropriate counterpart, which should then be assessed further (FAO/WHO, 2000b).

The issue of the potential occurrence of unintended effects due to the genetic modification process, such as the loss of existing traits or the acquisition of new ones, was examined. The occurrence of unintended effects is not unique for the application of recDNA techniques, but also occurs frequently in conventional breeding. Present approaches to detecting such effects focus on chemical analysis of known nutrients and toxicants (targeted approach). In order to increase the possibility of detecting unintended effects, profiling/fingerprinting methods are considered useful alternatives (non-targeted approach). This is of particular interest for plants with extensive modifications of the genome (second generation of genetically modified foods) where chances of the occurrence of unintended effects may increase.

Animal studies are deemed necessary to obtain information on the characteristics of newly expressed proteins, analogous to the conventional toxicity testing of food additives. Testing of whole foods may be considered if relevant changes in composition may have taken place in addition to the expected ones; however, such studies should be considered on a case-by-case basis, taking the limitations of this type of study into account. The minimum requirement to demonstrate the safety of long-term consumption of a food is a subchronic 90-day study. Longer-term studies may be needed if the results of a 90-day study indicate adverse effects such as proliferative changes in tissues.

The Expert Consultation noted that, in general, very little is known about the potential long-term effects of any foods, and that identification of such effects may be very difficult, if not impossible, due to the many confounding factors and the great genetic variability in food-related effects among the population. Thus the identification of long-term effects specifically attributable to genetically modified foods is highly unlikely. Epidemiological studies are not likely to identify such effects given the high

Table 1. Comparison of food safety regulations for genetic alterations of food crops

Nation	Legal act	Genetic alterations ^a				
		Insertion of genes (general)	Insertion of genes coding for previously approved gene products	Insertion of genes from same plant species (self-cloning)	Cross between approved transgenic lines	Mutation breeding and somaclonal variation (non GMO)
Australia ^b	ANZFA Food Standard A18	+	-	+	- ^c	-
Canada ^d	Food and Drug Act	+	+	+	(+)	+
EU ^e	Regulation 258/97/EC	+	+	+	+	(+)
Japan ^f	Food Sanitation Law	+	+	-	+	-
New Zealand ^b	ANZFA Food Standard A18	+	-	+	- ^g	-
USA ^h	FFDCA	+	-	(+)	(+)	(+)

^a+, To be evaluated; (+), should be evaluated unless substantially equivalent; -, evaluation not required.

^bANZFA, Australia–New Zealand Food Authority: ANZFA (1998).

^cNotification required: OGTR (2001).

^dHealth Canada (1994).

^eEU (1997a); EU (1997b); EU (1990).

^fMHW (2001).

^gThe New Zealand Hazardous Substances and New Organisms Act 1996 does not specifically provide for the breeding of approved genetically modified plant lines; however, the Australian Gene Technology Act 2000 does allow for this as "dealings" with GMOs: Australia (2000); New Zealand (1996).

^hFFDCA, Federal Food, Drug, and Cosmetic Act: FDA (1992); Maryanski (1995).

background of undesirable effects of conventional foods. The Consultation was of the opinion that pre-market safety assessment already gives an assurance that genetically modified foods are as safe as their conventional counterparts. Experimental studies, such as randomized controlled human trials, if properly performed, might provide additional evidence for human safety in the medium to long term.

At the FAO/WHO Expert Consultation on Allergenicity held in Rome in January 2001, a new decision tree was developed (FAO/WHO, 2001). The new decision tree builds on the one developed by ILSI/IFBC (Metcalf *et al.*, 1996) and FAO/WHO (1996).

When the source of the new protein is known to be allergenic, sequence similarity with known allergens and subsequent specific *in vitro* screening in sera from patients allergic to the source is recommended. Criteria for a positive outcome of sequence similarity include >35% identity in amino acid sequence between the expressed protein and a known allergen, or identity of six contiguous amino acids. In contrast to the previous decision trees, the new tree makes no distinction between commonly and less commonly allergenic sources with respect to *in vitro* screening. Any positive outcome defines the product as allergenic, and further product development should be discontinued. A negative outcome of the specific *in vitro* serum screening will lead to further targeted serum screening, testing the expressed protein for pepsin resistance and immunogenicity in animal models. Targeted

in vitro serum testing is done with sera from patients allergic to materials that are broadly related to the source of the original gene. Human *in vivo* testing may be considered in selected cases, but is not mandatory.

In case the new protein comes from a source not known to be allergenic, the decision-tree approach focuses on sequence similarity with known allergens, and subsequent targeted *in vitro* serum screening to test for cross-reactivity. Where there is sequence homology and the outcome of the serum-screening tests is positive, the protein is considered to be allergenic. Where the outcome is negative, further testing of the pepsin resistance of the new protein and immunogenicity testing in animal models may give indications for high or low probability of the allergenic potential of the new protein.

Food safety regulations

There is generally consistency in the national approaches to evaluating the food safety of genetically modified plants, as reviewed recently (Mackenzie, 2000). These approaches concur with those formulated by international consensus; however, there are some differences between Australia and New Zealand, Canada, the EU, Japan and the USA, as summarized in Table 1.

The safety evaluation may focus on different levels of the food crop, for example, the whole crop; crop tissues; or purified products, depending on the scope of the applica-

tion. In addition to foods and food ingredients, the use of purified products as food additives is also envisioned.

Food and food ingredients: European Union. In 1997 the Regulation on novel foods and novel food ingredients came into force in the European Union (EU, 1997a; EU 1997b). This regulation distinguishes six categories of novel food products, two directly referring to products derived from GMOs. The concept of substantial equivalence is fully endorsed in the European approach. It is stated that the assessment of substantial equivalence is an analytical process, where the novel food is compared to the most appropriate approved food, not necessarily meaning a conventional food, but possibly an earlier approved genetically modified variety. This analytical comparison to assess whether or not a novel food product is substantially equivalent to a product that is already on the market is, at the same time, the basis for both toxicological and nutritional assessments. If additional *in vivo* experiments are deemed necessary, it is stated to be essential to have sufficient knowledge on the nutritional characteristics of the novel food, for example, the energy content, protein content, and bioavailability of micronutrients. The highest test dosage should be the maximum amount of novel food product that can be included in a balanced animal diet, while the lowest dosage should be comparable to the expected amount in the human diet. If desirable safety factors cannot be reached in this way, additional investigations on resorption and metabolism of the novel food in animals, and eventually humans, are required; however, in specific cases lower safety factors may be acceptable if additional data show the safety of the novel food. The exposure assessment should include specific vulnerable consumer groups. For the nutritional assessment, it may be necessary in some cases to set up post-launch monitoring programmes. Also, with relation to allergenicity, the EU largely follows international consensus reports in that potential allergenicity should be investigated with the available means, to avoid the introduction of new allergens into the food supply. Thirteen decision trees are added to the regulation in order to guide producers to the data needed to establish the safety of an individual novel food product.

Food and food ingredients: international. Outside the EU, foods from genetically modified crops fit into regulatory frameworks that differ from nation to nation. Under Canadian regulations, genetically modified crops are considered novel foods, similarly to the EU (MacKenzie, 2000). Japanese and Australia/New Zealand's regulations, on the other hand, focus specifically on foods derived from genetically modified crops (ANZFA, 1998; MacKenzie, 2000). The American regulations do not in principle regard genetically modified crops as a separate entity with

respect to other foods. Rather, the focus is on the altered characteristics brought about by genetic modification, and the intended use of the novel crop (FDA, 1992).

Food additives. Food additives derived from GMOs are regulated differently in Australia/New Zealand, Canada, the EU, the USA and Japan, and the definition of 'food additive' varies between these nations. In the EU and Canada, the evaluation of food additives – non-nutrient substances not conventionally present in food – does not distinguish between food additives derived from GMOs or from other sources. In Australia and New Zealand, food additives from GMOs are evaluated for the components that deviate from the existing specifications for food additives. In the USA, a food additive is defined as a non-GRAS (non-'generally recognized as safe') food component. Food components that are 'food additives' under EU legislation may therefore be considered either food ingredients (GRAS) or American 'food additives'. Commercial use of American food additives requires a permit following a safety evaluation by the Food and Drug Administration (FDA). Introduced gene products are considered food-additives, i.e. non-GRAS components, unless they have already been declared GRAS (FDA, 1992).

In Japan, both genetically modified foods and food additives are subject to the same evaluation procedure (MHW, 2001).

The concept of substantial equivalence and its practical implications

The safety of our existing foods is based on long-term experience and history of safe use, even though they may contain anti-nutritional or toxic substances (OECD, 1993a). In the past decades progress has been made with respect to identification and characterization of food constituents which may exert adverse and/or beneficial effects on chronic intake. Several compounds have been identified with anti-carcinogenic effects (saponins, glucosinolates, isoflavones), and positive effects on osteoporosis (isoflavones) and on the incidence of cardiovascular diseases (flavonoids), while certain plant compounds may also exert adverse mutagenic and carcinogenic effects (Essers *et al.*, 1998). In many cases beneficial compounds may also exert adverse effects, depending on the conditions and the presence of other agonists or antagonists. The scientific basis underpinning the relationship between food and its constituents and health is still fragmentary, but positive and negative effects due to the consumption of certain food constituents cannot be ignored. Thus the OECD concept of generally recognized safety of the existing food supply will undergo further refinement in the light of growing scientific evidence for the biological relevance of specific food constituents.

Table 2. Studies on the composition of genetically modified crops^a

Host plant	Trait	Parameter tested ^b	Reference
Canola	high lauric acid	AA, EA, FA, GL	Redenbaugh <i>et al.</i> (1995)
Canola GT73	herbicide resistant (glyphosate)	AA, EA, FA, GL, MI, PA, PX, SI	ANZFA (2000a)
Cotton 1445	herbicide resistant (glyphosate)	AA, FA, GP, MT, PX, TF	Nida <i>et al.</i> (1996) ANZFA (2000e)
Cotton	herbicide resistant (bromoxynil)	AA, CP, FA, GP	Redenbaugh <i>et al.</i> (1995)
Maize GA21	herbicide resistant (glyphosate)	AA, FA, MI, PX ANZFA (2000b)	Sidhu <i>et al.</i> (2000)
Maize	herbicide resistant (glufosinate)	AA, FA, PX, SU	Böhme and Aulrich (1999)
Maize	insect resistant (Cry1Ab)	AA, FA, MI, PX	Sanders <i>et al.</i> (1998)
Maize	insect resistant (Cry1Ab)	MT, PX	Masoero <i>et al.</i> (1999)
Maize Bt176	insect resistant (Cry1Ab)	AA, MT, PX	Brake and Vlachos (1998)
Maize Bt176	insect resistant (Cry1Ab)	AA, FA, MI, PX, SU	Aulrich <i>et al.</i> (1999)
Maize MON810	insect resistant (Cry1Ab)	AA, FA, MI, PA, PX, SU, TF, TI	ANZFA (2000c)
Potato	herbicide resistant (chlorsulfuron)	AA, PX	Conner (1994)
Potato	insect resistant (Cry3A)	GA, MI, PX, VI	Lavrik <i>et al.</i> (1995)
Rice	soybean glycinin	AA, FA, MI, PX, VI	Momma <i>et al.</i> (1999)
Soybean GTS 40-3-2	herbicide resistant (glyphosate)	AA, FA, IF, LE, PA, PX, SR, TI, UR	Padgett <i>et al.</i> (1996)
Soybean GTS 40-3-2	herbicide resistant (glyphosate)	IF	Lappé <i>et al.</i> (1999)
Soybean	high-oleic acid	AA, FA, IF, MI, PA, PX, SR, TI, VI	ANZFA (2000d)
Squash	virus resistant (ZYMV, WMV2)	MI, PX, SU, VI	Quemada (1996)
Sugar beet	herbicide resistant (glufosinate)	PX	Böhme and Aulrich (1999)
Tomato	insect resistant (Cry1Ab)	AA, MI, PX, TO, VI	Noteborn <i>et al.</i> (1995)
Tomato (Flavr Savr)	antisense polygalacturonase	MI, PR, TO, VI	Redenbaugh <i>et al.</i> (1991)

^aData from publicly available reports.

^bAbbreviations: AA, amino acids; CP, cyclopropenoid fatty acids; EA, erucic acid; FA, fatty acids; GA, glycoalkaloids; GL, glucosinolates; GP, gossypol; IF, isoflavones; LE, lectins; MI, minerals; MT, mycotoxins; PA, phytic acid; PR, protein; PX, proximates (e.g. protein, fat, ash, fibre, moisture, carbohydrate); SI, sinapine; SR, stachyose and raffinose; SU, sugars; TF, tocopherol(s); TI, trypsin inhibitor; TO, alpha-tomatins; UR, urease; VI, vitamins.

It is generally acknowledged that the basis for the food-safety evaluation of complex (plant) products should be a comparison with (i) the nearest comparator (in genetic terms); and (ii) other varieties that are already on the market, in that order. This comparison comprises phenotypic characteristics and composition. The breeder usually performs the phenotypic comparison, but the criteria are not well defined. On the other hand, this type of comparison has been used successfully in plant breeding for decades, and has led to many new varieties with virtually no negative consequences for the consumer.

The compositional analysis has been the subject of an ongoing discussion in many national and international meetings on the issue of the safety of GMO-derived products. Table 2 gives an account of genetically modified crops for which compositional data have been published. From the start of the practice of comparing constituents of genetically modified varieties with their traditional counterpart, it has been advocated that both nutrients and anti-

nutrients should be included in the analysis. As mentioned above, the OECD has taken the lead in formulating Consensus Documents (OECD, 2001a) which group constituents that should be analysed, in all cases, in any new variety of the given crop. Additional analyses may be required, depending on the type of genetic modification or in order to further investigate detected differences. This should be determined on a case-by-case basis. International harmonization and standardization are necessary to avoid differences in data requirements in different countries and thereby to prevent trade barriers. OECD Consensus Documents have now have been formulated for soybean and rapeseed, while others on maize, potato, sugar beet and rice are in the pipeline.

The comparator for assessment of substantial equivalence will preferably be the direct parent line, which will not, however, be available in all cases. If the parent line is not available for comparison, the OECD advocates the use of several control lines to determine whether any observed

differences may arise from secondary effects from the genetic alteration (OECD, 1993a). Compositional analyses should be performed on genetically modified plants and their comparators that have been grown under similar environmental conditions, as these conditions may lead to (large) differences in composition that are not related to the genetic modification. At the same time, it is deemed necessary to assess the novel genetically modified variety at different locations (different environmental conditions) and during subsequent growing seasons (different climatological conditions) in order to assess whether other metabolic pathways may be turned on or switched off under different conditions, with possible (adverse) effects on the composition of the food plant. The number of environmentally different locations where the genetically modified plant needs to be assessed is, in most cases, not specified. 'Standard' statistical analyses are usually performed on data for the genetically modified variety and the parent line, leading to acceptance or rejection of the hypothesis with a certain probability. However, it is feasible that this system will need to be further elaborated in the (near) future, as different aberrant compositional profiles may be acceptable for different groups of constituents. It can be envisioned that significant changes in metabolic pathways leading to toxic plant substances, such as glycoalkaloids in potato and tomato, will need to be investigated further – even if the changes do not lead to natural toxin levels that fall outside the natural variability ranges, as documented in the literature or determined from a traditionally bred group of control varieties. On the other hand, it should be clear that in those cases where differences in composition between the modified organism and its counterpart fall outside these ranges of variability, such crops do not necessarily pose a threat to human health. Whether additional investigations are appropriate to address any further concerns related to the food safety of the crop plant should be assessed on a case-by-case basis. The Nordic Council proposed that, in the case of a difference of 20% in the average value for the new plant variety compared to the parent line, an explanation should be sought (Nordic Council, 2000); but it is doubtful whether acceptable degrees of compositional differences can be defined in general.

Risk assessments: what they are and how they are done

Safety assessment of food additives and food contaminants

The safety or risk evaluation of food additives, residues of pesticides and veterinary drugs, and food contaminants is based on (i) hazard identification; (ii) hazard characterization; and (iii) assessment of exposure. Hazard is defined as the potential of a chemical agent to cause harmful effect(s),

and risk as a function of the probability that an adverse effect will occur due to the presence of a hazardous compound in food and the severity of the adverse effect (exposure \times toxicity). FAO/WHO and the International Program on Chemical Safety (IPCS) have developed strategies for the safety evaluation of these types of chemicals which may be present in food (WHO, 1987; WHO, 1990). These strategies focus on the establishment of a level of daily intake by humans, on a body weight basis, which would not cause an appreciable risk (acceptable daily intake, ADI). The assumption is that for most toxic effects induced by chemicals, a threshold level can be determined, that is, a dose level below which a toxic effect is not apparent. In order to arrive at such a dose (no observed adverse effect level, NOAEL), a battery of standardized toxicity tests is carried out: acute and (sub)-chronic toxicity studies, genotoxicity studies, carcinogenicity studies, and specific studies concerning immunotoxicity, reproduction and developmental toxicity. The protocols for such studies have been elaborated by OECD (1993b).

From these studies, mostly carried out in laboratory animals, the NOAEL is determined and, upon application of a safety factor, the ADI is derived. In many cases a safety factor of 100 is used, allowing for differences in sensitivity between test animals and humans and for differences within the human population. Depending on the available data and the substance under study higher or lower safety factors may be applied. The use of relatively large default factors in establishing an ADI probably provides an overestimation of the true risk involved, and can be considered as a 'safety first' approach. However, certain chemicals such as genotoxic carcinogens do not show a dose-dependent threshold level – in these cases the ADI concept is not applicable, and a quantitative risk assessment is carried out taking into account the incidence of DNA damage and tumours versus the applied dose.

Safety assessment of whole foods

As whole foods contain mixtures of macro- and micro-nutrients, anti-nutrients and plant toxins, the safety evaluation of foods as described above for single, well defined chemicals is virtually impossible. Foods may contain toxic compounds, often with small margins of safety between actual intake and apparent toxic-effect levels. For instance, the safety margin for potato glycoalkaloids may be between 2 and 6, assuming a lowest observed effect level in humans of 2 mg kg⁻¹ body weight, an average level of glycoalkaloids in potato of 200 mg kg⁻¹, and an average daily consumption of 300 g (Nordic Working Group, 1991). Moreover, for certain micronutrients margins may be small between levels that are beneficial or essential for human health, and levels that are toxic. For instance, the recommended dose of vitamin A is

1 mg day⁻¹ for pregnant women, while the estimated safe daily intake level is 3 mg, and teratogenic effects have been observed at a daily intake of 7.5 mg day⁻¹ (Rothman *et al.*, 1995). In the case of essential amino acids, adverse effect levels in mg kg⁻¹ body weight are only three to four times the nutritional requirements in humans (IFBC, 1990). Application of the usual safety factor of 100 would result in inadequate nutritional levels.

Testing of whole foods in laboratory animals has its specific problems, and considerable experience has been gained with toxicological testing of irradiated foods (Hammond *et al.*, 1996a). The amounts of foods to be administered to animals are limited due to effects on satiety and possible negative interference with the nutritional balance of the animal diet. Feeding animals with whole foods at exaggerated dose levels may induce a series of adverse effects that would mask potential adverse effects caused by alterations induced by the genetic modification. The highest test dosages should be the maximum amount of novel food product that can be included in a balanced animal diet, while the lowest dosage should be comparable to the expected amount in the human diet. Furthermore, the bio-availability (uptake, metabolism and kinetics) of food constituents may be different when ingested as part of the food matrix.

The minimum duration of an animal study with whole foods to demonstrate the safety of long-term consumption of a food depends on the available toxicity database for the food under investigation. The 2000 FAO/WHO Expert Consultation (FAO/WHO, 2000b) recommended as minimum requirement a subchronic 90-day study, with possibly longer-term studies needed if the results of a 90-day study indicate adverse effects, such as proliferative changes in tissues. Further studies are needed to establish specific research protocols.

Appropriate safety testing with whole foods should be hypothesis-driven and should be carried out in parallel with toxicity studies on specific, isolated food constituents. A combination of animal experiments, *in vitro* experiments with tissues and/or organs from animals and humans, and possibly human clinical studies, should be carried out, focusing on the identification of biomarkers for exposure and markers that are predictive in an early phase of exposure of chronic toxicity (Diplock *et al.*, 1999).

Exposure assessment and role of diet

For both traditionally bred and genetically modified varieties with a novel trait that either intentionally or unintentionally interferes with the nutritional characteristics of the crop, it will be very important to assess adequately the consequences of introducing this novel plant variety onto the market, for the nutritional status of specific consumer groups and the entire population. In

order to do this effectively and identify possible consumer groups at risk of nutritional deficiencies, it will be necessary to have detailed information on the consumption patterns of different consumer groups within the population, and on geographic variations in these patterns. Extensive databases are currently available only for a limited number of (areas within) countries. The availability of such databases will become more compelling when more novel foods entering the market have significantly altered nutritional characteristics. New models to assess the exposure of individual consumers to individual foods and food ingredients will also gain importance. An example of such an approach is the Monte Carlo simulation approach.

Monte Carlo models assess the distribution of exposure of individuals within a given population, taking into account the probability that exposures from more than one source (food product) may occur on a single day without overstating the actual exposure. This is especially relevant for both novel transgenic proteins that are introduced in different food crops and/or crop varieties, as well as for GMO-derived ingredients that may be used in a large variety of food products. To obtain reliable information from models of this type, it is necessary to collect sufficiently sound input parameters for the populations under investigation.

Experiences with risk assessment of genetically modified food crops

Safety evaluation of newly expressed proteins

If substantial equivalence can be established except for a single or few specific traits of the genetically modified plant, further assessment focuses on the newly introduced trait itself (EU, 1997b). Demonstration of the lack of amino acid sequence homology to known protein toxins/allergens, and a rapid proteolytic degradation under simulated mammalian digestion conditions, was deemed to be sufficient to assume the safety of the new protein (FAO/WHO, 1996). However, there may be circumstances that require more extensive testing of the new protein, such as (i) the specificity and biological function/mode of action of the protein is partly known or unknown; (ii) the protein is implicated in mammalian toxicity; (iii) human and animal exposure to the protein is not documented; or (iv) modification of the primary structure of naturally occurring forms. Bacterial Bt proteins are an example of proteins that have been introduced into crop varieties by genetic modification.

Bt proteins (Cry proteins) from *Bacillus thuringiensis* strains have been introduced into genetically modified crop plants for their insecticidal properties in the larvae of target herbivorous insect species (Peferoen, 1997). The

Table 3. Toxicity studies of proteins expressed in commercialized genetically modified crops^a

Transgene product	Tests ^{b,c}								
	SC	ID	AO	AI	SO	SE	IR	HP	BI
Acetolactate synthase (<i>Arabidopsis thaliana</i>)	1								
12 : 0 Acyl carrier protein thioesterase (<i>Umbellularia californica</i>)	2	2	2						
1-Aminocyclopropane-1-carboxylic acid deaminase (<i>Pseudomonas chloroaphis</i>)	3	3							
Barnase (<i>Bacillus amyloliquefaciens</i>)	4								
Barstar (<i>Bacillus amyloliquefaciens</i>)	4								
Beta-glucuronidase (<i>Escherichia coli</i> K12)	5	5	5						
Bromoxynil nitrilase (<i>Klebsiella pneumoniae</i> var. <i>ozaenae</i>)	6	7							
Coat protein (cucumber mosaic virus)	8								
Coat protein (potato virus Y)	9								
Coat protein (watermelon mosaic virus 2)	8								
Coat protein (zucchini yellows mosaic virus)	8								
Cry1Ab endotoxin (<i>Bacillus thuringiensis</i> var. <i>kurstaki</i>)	10	11	12	13	11			11	11
Cry1Ac endotoxin (<i>Bacillus thuringiensis</i> var. <i>kurstaki</i>)	14	12	12				15		16
Cry1F endotoxin (<i>Bacillus thuringiensis</i> var. <i>aizawai</i>)	17	17	17						
Cry3A endotoxin (<i>Bacillus thuringiensis</i> var. <i>tenebrionis</i>)	18	12	12						
Cry9C endotoxin (<i>Bacillus thuringiensis</i> var. <i>tolworthi</i>)	13	13	13	13	13	13			13
5-Enolpyruvylshikimate-3-phosphate synthase (<i>Agrobacterium</i> sp. CP4)	19	19	19						
5-Enolpyruvylshikimate-3-phosphate synthase (<i>Zea mays</i>)	20	20	20						
Glyphosate oxidoreductase (<i>Ochromobactrum anthropii</i> LBAA)	21	21	21						
Neomycin phosphotransferase II (<i>Escherichia coli</i> Tn5)	4	22	22						
Phosphinothricin acetyltransferase (<i>Streptomyces hygrosopicus</i> , <i>bar</i> gene)	4	23	14						
Phosphinothricin acetyltransferase (<i>Streptomyces viridochromogenes</i> , <i>pat</i> gene)	24	23	25						
Replicase (potato leaf roll virus)	26								

^aData from publicly available reports.

^bAO, acute oral toxicity, rodent, gavage; AI, acute intravenous toxicity, rodent, single dose; BI, binding to mammalian intestinal tissues; HP, haemolytic potential; ID, *in vitro* digestion; IR, immune response, rodent; SC, sequence comparisons with allergens and toxins; SE, sensitization, oral and intraperitoneal, rodent; SO, subchronic oral toxicity, 4-week, rodent.

^cReferences: **1** flax Cdc Triffid Fp967, 1999 (Health Canada, 2001); **2** canola, high-laurate, DD96-08 (CFIA, 2001); **3** Reed *et al.* (1996); **4** canola MS1 ×RF1, DD95-04 (CFIA, 2001); **5** EPA (2000c); **6** Bxn plus Bt cotton, 2000 (Health Canada, 2001); **7** canola Westar-oxy-235, 1997 (Health Canada, 2001); **8** Squash Czw-31999 (Health Canada, 2001); **9** potato lines SEMT15-02 etc., 1999 (Health Canada, 2001); **10** ANZFA (2000c); **11** Noteborn *et al.* (1995); **12** FIFRA SAP (2000a); **13** FIFRA SAP (2000b); **14** maize DBT418, 1997 (Health Canada, 2001); **15** Vazquez Padron *et al.* (1999); Vazquez *et al.* (1999); **16** Vazquez Padron *et al.* (2000); **17** EPA (2000d); **18** potato lines ATBT04-6 etc., 1999 (Health Canada, 2001); **19** Harrison *et al.* (1996); **20** ANZFA (2000b); **21** ANZFA (2000a); **22** Fuchs *et al.* (1993); **23** Wehrmann *et al.* (1996); **24** canola HCN92, DD95-01 (CFIA, 2001); **25** maize T14 and T25, 1997 (Health Canada, 2001); **26** potato lines RBMT21-129 etc., 1999 (Health Canada, 2001).

working mechanism is based on specific receptor binding in susceptible insect larvae in epithelial cells of the midgut, leading to pore formation, cell lysis, disintegration of the epithelium lining in their midgut, and eventually to death of the larvae due to starvation. This type of biological action of the newly introduced protein directs further toxicity testing in mammals. A general drawback is that newly expressed pesticidal proteins, such as Bt toxins and lectins, are often present in the genetically modified plant at levels too low for extensive testing. Therefore, sufficient amounts of the new proteins are obtained from cultures of overexpressing bacterial strains. This carries the potential hazard that toxic impurities can be present, and that protein processing, like glycosylation, may be different in plants and bacteria. Therefore it is important to demonstrate that production in an alternative host does not result in differences in toxicity. For these pesticidal proteins, the

following properties must be comparatively investigated: (i) electrophoretic behaviour of full-length as well as trypsinated forms; (ii) immunoreactivity with poly- and/or monoclonal antibodies; (iii) identical patterns of post-translation modification; (iv) sequence similarity; and (v) functional characteristics to target insect species.

The safety of a number of newly inserted proteins has been tested on a case-by-case basis (Table 3). It should be noted that for transgenic viral proteins in crops approved in Canada and the USA, their consumption has been assumed to be safe based on the history of ingestion of the wild-type plant viruses contained within plant foods.

In the case of the Cry1Ab5 and Cry9C proteins, various studies have been performed on binding to tissues of the gastro-intestinal tract of rodents and primates, including humans (EPA, 2000a; Noteborn *et al.*, 1995). There is no evidence for the presence of specific receptors in mam-

malian tissues for these proteins, nor are there indications of an amino acid sequence homology to known protein toxins/food allergens. A number of toxicity tests have been performed with respect to:

digestibility and stability in *in vitro* simulated gastric and intestinal fluids and *in vivo* models;

acute oral toxicity in a rodent species;

subchronic toxicity (30-day repeated-dose feeding) with focus on a tier I immunotoxicity screening.

Experiments performed with single and repeated dosing of the Cry proteins Cry1Ab5 and Cry9C, at levels up to 10 000 times those produced in genetically modified plants, did not indicate toxic effects in the rat, and histopathological analysis did not show binding of the Cry proteins to the intestinal epithelium of rodents and tissues of other mammals. In contrast to Cry1Ab5, Cry9C showed resistance to proteolysis under simulated human gastric conditions (pH > 2.0) and denaturation at elevated temperatures. On the other hand, it was noted that Cry9C degraded completely upon pepsin treatment at pH < 1.5 (human 'fasting' values). However, the digestibility of protein preparations under simulated conditions is of limited value, as questions can be raised as to whether these assays do mimic the physiological state of human beings.

In cases of (i) a completely novel gene; (ii) novel proteins as anti-nutrients; (iii) novel proteins without a clear threshold (bacterial toxins); (iv) predicted high levels of intake of toxic proteins such as protease inhibitors; and (v) non-rapidly degradable proteins, more extensive toxicity testing with the pure protein at exaggerated doses may be required.

Safety evaluation of whole genetically modified foods

Examples of feeding studies with whole genetically modified foods are summarized in Table 4. In the case of the Bt tomato experiment, a semi-synthetic rodent diet was supplemented with 10% (w/w) of lyophilized genetically modified or control tomato powder, and fed during 91 days. The average daily intake was approximately 200 g tomato day⁻¹ per rat, corresponding to a daily human consumption of 13 kg. No clinical, toxicological or histopathological abnormalities were observed. The 10% (w/w) tomato content of the diet was chosen because of the relatively high potassium content of tomato (40–60 g kg⁻¹), while higher amounts could have caused renal toxicity (Noteborn and Kuiper, 1994).

Fares and El Sayed (1998) reported that mice fed for 14 days on fresh potato immersed in a suspension of delta-endotoxin of *B. thuringiensis* var. *kurstaki* strain HD1 developed an increase of hyperplastic cells in their ileum. Feeding with fresh genetically modified potato expressing the *cry1* gene caused mild adverse changes in the various

ileac compartments, as compared to the control group on fresh potato. The occurrence of these effects in mice fed either 'spiked' potato or genetically modified potato may have been due to the toxicity of the Cry1 protein; however, no details were given on the intake of Cry1 protein or on dietary composition, which limits interpretation of this study.

Following the short-term safety assessment of transgenic potato and rice with native and designed soybean glycinin (four additional methioninyl residues), Hashimoto *et al.* (1999a); Hashimoto *et al.* (1999b) and Momma *et al.* (2000) demonstrated that a daily administration of 2.0 g potato and 10 g rice kg⁻¹ body weight to rats for 4 weeks indicated neither pathological nor histopathological abnormalities in liver and kidney.

The experiments reported by Ewen and Pusztai (1999) indicated, according to the authors, that rats fed genetically modified potato containing GNA lectin showed proliferative and antiproliferative effects in the gut. These effects are presumed to be due to alterations in the composition of the transgenic potato, rather than to the newly expressed gene product; however, various shortcomings of this study, such as the protein deficiency of the diets and the lack of control diets, make the results difficult to interpret (Kuiper *et al.*, 1999). Similar criticisms have been made by the UK's Royal Society (Royal Society, 1999).

Teshima *et al.* (2000) fed Brown Norway rats and B10A mice with either heat-treated genetically modified soybean meal containing the *cp4-epsps* gene, or control non-genetically modified soybean meal. These experimental animals were employed based on their immunosensitivity to oral challenges. The semi-synthetic animal diet was supplemented with 30% (w/w) heat-treated soybean meal, and fed over 105 days. Both treatments failed to cause immunotoxic activity or to cause the IgE levels to rise in the serum of rats and mice. Moreover, no significant abnormalities were observed histopathologically in the mucosa of the small intestine of animals fed either genetically modified or non-genetically modified soybean.

In addition to the feeding studies described above, studies have been performed on domestic animals fed genetically modified crops to establish performance (feed conversion; Table 5). It is apparent that no harmonized design exists yet for feeding trials in animals to test the safety of genetically modified foods.

Allergenicity

The potential allergenicity of newly introduced proteins in genetically modified foods is a major safety concern. This is true in particular for genetic material obtained from sources with an unknown allergenic history, such as the soil bacterium *B. thuringiensis*. An illustrative case of a

Table 4. Toxicity studies performed with genetically modified food crops^a

Crop	Trait	Species	Duration	Parameters	Reference
Cottonseed	Bt endotoxin (<i>Bacillus thuringiensis</i>)	rat	28 days	body weight feed conversion histopathology of organs blood chemistry	Chen <i>et al.</i> (1996)
Maize	Cry9C endotoxin (<i>Bacillus thuringiensis</i> var. <i>tolworthi</i>)	human		reactivity with sera from maize-allergic patients	EPA (2000e)
Potato	lectin (<i>Galanthus nivalis</i>)	rat	10 days	histopathology of intestines	Ewen and Pusztai (1999)
Potato	Cry1 endotoxin (<i>Bacillus thuringiensis</i> var. <i>kurstaki</i> HD1)	mouse	14 days	histopathology of intestines	Fares and El Sayed (1998)
Potato	glycinin (soybean, <i>Glycine max</i>)	rat	28 days	feed consumption body weight blood chemistry blood count organ weights liver- and kidney- histopathology	Hashimoto <i>et al.</i> (1999a) Hashimoto <i>et al.</i> (1999b)
Rice	glycinin (soybean, <i>Glycine max</i>)	rat	28 days	feed consumption body weight blood chemistry blood count organ weights liver- and kidney- histopathology	Momma <i>et al.</i> (2000)
Rice ^b	phosphinothricin acetyltransferase (<i>Streptomyces hygroscopicus</i>)	mouse, rat	acute and 30 days	feed consumption body weight median lethal dose blood chemistry organ weight histopathology	Wang <i>et al.</i> (2000)
Soybean GTS 40-3-2	CP4 EPSPS (<i>Agrobacterium</i>)	rat, mouse	105 days	feed consumption body weight histopathology of intestines and immune system serum IgE and IgG levels	Teshima <i>et al.</i> (2000)
Soybean GTS 40-3-2	CP4 EPSPS (<i>Agrobacterium</i>)	human		reactivity with sera from soybean-allergic patients	Burks and Fuchs (1995)
Soybean GTS 40-3-2	CP4 EPSPS (<i>Agrobacterium</i>)	rat	150 days	blood chemistry urine composition hepatic enzyme activities	Tutel'yan <i>et al.</i> (1999)
Soybean	2S albumin (Brazil nut, <i>Bertholletia excelsa</i>)	human		reactivity with sera from Brazil nut-allergic patients	Nordlee <i>et al.</i> (1996)
Tomato	Cry1Ab endotoxin (<i>Bacillus thuringiensis</i> var. <i>kurstaki</i>)	rat	91 days	feed consumption body weight organ weights blood chemistry histopathology	Noteborn <i>et al.</i> (1995)
Tomato	antisense polygalacturonase (tomato, <i>Lycopersicon esculentum</i>)	rat	28 days	feed consumption body weight organ weights blood chemistry histopathology	Hattan (1996)

^aData from publicly available reports.^bMutagenicity additionally tested.

genetically modified food for which the allergenic risk has to be assessed is maize in which the truncated Cry9C protein (MW 68 kDa) is expressed, and which has been

allowed as a transgene product in StarLink yellow maize for animal feed in the USA (EPA, 2000a). It should be noted that the protoxin Cry9C from *B. thuringiensis* var. *tolworthi*

Table 5. Performance studies on animals fed genetically modified crops^a

Crop	Trait	Animal	Parameters	Duration	Reference
Canola GT73, meal	herbicide resistant	quail	weight increase feed consumption mortality	5 days	ANZFA (2000a)
Canola GT73, meal	herbicide resistant	trout	weight increase	70 days	ANZFA (2000a)
Maize GA21, kernel	herbicide resistant	broiler chicken	weight increase feed consumption fat pads	40 days	Sidhu <i>et al.</i> (2000)
Maize CBH351, kernel	insect resistant	broiler chicken	weight increase feed consumption breast muscle weight fat pads weight mortality	42 days	EPA (2000f)
Maize, kernel	herbicide resistant	swine	feed conversion	8 days	Böhme and Aulrich (1999)
Maize Bt176, kernel	insect resistant	broiler chicken	weight increase feed consumption organ weights	41 days	Brake and Vlachos (1998)
Maize Bt176, kernel	insect resistant	broiler chicken	feed consumption feed conversion	35 days	Aulrich <i>et al.</i> (1999)
Maize Bt176, kernel	insect resistant	laying hen	feed consumption egg production feed conversion	10 days	Aulrich <i>et al.</i> (1999)
Maize Bt176, silage	insect resistant	sheep	feed conversion	?	Aulrich <i>et al.</i> (1999)
Maize Bt176, silage	insect resistant	beef steer	weight increase feed conversion meat yield	246 days	Aulrich <i>et al.</i> (1999)
Soybean GTS 40-30-2, raw	herbicide resistant	lactating cow	body weight milk production milk composition dry matter digestibility ruminal fluid composition	29 days	Hammond <i>et al.</i> (1996b)
Soybean GTS 40-30-2, meal	herbicide resistant	broiler chicken	weight increase feed consumption breast muscle weight fat pads weight mortality	42 days	Hammond <i>et al.</i> (1996b)
Soybean GTS 40-30-2, meal	herbicide resistant	channel catfish	weight increase feed consumption filet composition	70 days	Hammond <i>et al.</i> (1996b)
Soybean, meal	high oleic acid	swine	weight increase feed consumption	17 days	ANZFA (2000d)
Soybean, meal	high oleic acid	broiler chicken	weight increase feed consumption	18 days	ANZFA (2000d)
Sugar beet, beet	herbicide resistant	swine	feed conversion	8 days	Böhme and Aulrich (1999)

^aData from publicly available reports.

has been modified at residue 164 by substituting the arginine residue with lysine to increase serine protease resistance in the field (Lambert *et al.*, 1996). Recent investigations have found traces of the *Cry9C* gene and/or protein in taco shells (CNN, 2000; EPA, 2000a). The *Cry9C* protein has also been detected in maize seeds of a non-StarLink variety or in maize from such seeds (FDA, 2000). This has raised the issue of potential allergenicity of the genetically modified maize for humans. *Cry9C* might be a potential allergen because the protein shows some characteristics of known food allergens: (i) an MW of 68 kDa;

(ii) relative resistance to gastric proteolytic degradation and to heat and acid treatment; (iii) it is probably a glycoprotein; (iv) induces a positive IgE response in the Brown Norway rat, and is a high IgE responder on intraperitoneal and oral sensitization, in contrast to the related *Cry1Ab5* protein; and (v) may be found intact in the bloodstream after oral feeding in a rat model. On the other hand, *Cry9C* has no amino acid sequence homology to any known allergen or protein toxin, and wild-type and StarLink maize protein extracts have been demonstrated to be indistinguishable in their reactivities towards sera of

maize-allergic and major food-allergic patients. Furthermore, no immunogenic/toxic effects were observed in a 30-day repeated-dose study in mice with Cry9C (EPA, 2000b), and the bioavailability of the protein in the rat is relatively low (0.0002–0.0006%), which reduces the likelihood of sensitization.

Levels of Cry9C in maize-derived food products appear to be much less than the >1% level apparently characteristic of food allergens (10–80%). Post-harvest blending and mixing may have diluted the Cry9C protein in food products to the p.p.b. range for the harvest years 2000 and 1999. Maize in food channels is either wet-mill processed, which produces high-fructose corn syrup, glucose, dextrose, starch or oil; or dry-milled, which produces primarily cereals, flour and meal. A preliminary study using Cry9C ELISA well tests showed that there was no intact Cry9C protein in a limited number of starch samples (EPA, 2001). In this study no other wet-milling products were assayed, and the ELISA was not validated for detection of Cry9C in starch. In general, the protein fraction goes to feed use (FIFRA SAP, 2000b). Upon dry-milling, the Cry9C protein content is reduced by 40%. Additional processing, such as alkaline cooking (masa production), decreases the protein content to 0.1–0.2% of the original Cry9C protein (FIFRA SAP, 2000b). This suggests a further reduction in allergenic potency; however, protein denaturation by heat or partial proteolysis may uncover new allergenic epitopes (FIFRA SAP, 2000b; Hefle, 1996). It is therefore important to note the need for reproducible, validated methods for analysing Cry9C protein levels in processed foods and intermediates, as distinct from the PCR methods (CDC, 2001a; EPA, 2001). The estimated duration of exposure to Cry9C is uncertain, but may have been too short to promote sensitization and induce allergic reactions.

After the media (CNN, 2000) reported the inadvertent introduction of StarLink maize into the food supply, some consumers reported adverse health effects consistent with allergic reactions after eating maize products, or from another cause (FIFRA SAP, 2000b). Subsequently the FDA, with the assistance of the Centers for Disease Control and Prevention (CDC), evaluated 28 consumer complaints linked to foods allegedly containing StarLink maize. Analysis by ELISA revealed, however, that the banked serum samples did not contain Cry9C-specific IgE antibody (CDC, 2001b).

Although reassuring, these follow-up studies of FDA/CDC's reported putative illnesses linked to StarLink maize are not conclusive as yet. The FDA's IgE-specific ELISA did not include the StarLink-derived Cry9C protein, but the recombinant Cry9C expressed in *Escherichia coli* as antigen. Consequently, it is possible that epitopes present on Cry9C in maize may not be present in the non-glycosylated *E. coli*-derived protein. It is also recognized that a specific

goat antiserum against Cry9C was included in the ELISA, as there was no human serum available that contained the IgE antibody to Cry9C. The result is that the possibility of lack of specificity for human anti-Cry9C IgE cannot be entirely dismissed (CDC, 2001b; CDC, 2001c). The StarLink yellow maize case highlights the difficulty that there can be no final proof as to whether Cry9C is, or is not, a food allergen.

An example of a transgene from an allergenic source is that of the Brazil nut (*Bertholletia excelsa*) 2S albumin expressed in soybean. This protein is rich in methionine, and would therefore increase the nutritive value of soybean for animal feed. It was found, however, that the transgenic protein was reactive towards sera from patients who were allergic to Brazil nut, and the further development of this soybean was halted (Nordlee *et al.*, 1996).

Detection and characterization of unintended effects

Upon random insertion of specific DNA sequences into the plant genome (intended effect), the disruption, modification or silencing of active genes or the activation of silent genes may occur, which may result in the formation of either new metabolites or altered levels of existing metabolites. Unintended effects may be partly predictable on the basis of knowledge of the place of the transgenic DNA insertion, the function of the inserted trait, or its involvement in metabolic pathways; while other effects are unpredictable due to the limited knowledge of gene regulation and gene–gene interactions (pleiotropic effects). It should be emphasized that the occurrence of unintended effects is not specific for genome modification through recDNA technology – it also occurs frequently in conventional breeding. Unintended effects may be identified by an analysis of the agronomical/morphological characteristics of the new plant and an extensive chemical analysis of key nutrients, anti-nutrients and toxicants typical for the plant. Limitations of this analytical, comparative approach are the possible occurrence of unknown toxicants and anti-nutrients, in particular in food plant species with no history of (safe) use; and the availability of adequate detection methods.

Examples of unexpected secondary effects due to either somaclonal variations, pleiotropic effects or genetic modification, which may be of biological or agronomic importance to the plant, are illustrated in Table 6. Some of these alterations would indicate that the experimental, genetically modified plant does not possess the appropriate properties to allow further development into a commercial crop plant. Others would be identified only through appropriate field trials (e.g. soybean; Gertz *et al.*, 1999). In order to identify potential secondary effects of the genetic modification, which would result in alterations in the composition of genetically modified crops, different strategies may be applied, for example the targeted (com-

Table 6. Unintended effects in genetic engineering breeding^a

Host plant	Trait	Unintended effect	Reference
Canola	overexpression of phytoene-synthase	multiple metabolic changes (tocopherol, chlorophyll, fatty acids, phytoene)	Shewmaker <i>et al.</i> (1999)
Potato	expression of yeast invertase	reduced glycoalkaloid content (-37–48%)	Engel <i>et al.</i> (1998)
Potato	expression of soybean glycinin	increased glycoalkaloid content (+16–88%)	Hashimoto <i>et al.</i> (1999a); Hashimoto <i>et al.</i> (1999b)
Potato	expression of bacterial levansucrase	adverse tuber tissue perturbations; impaired carbohydrate transport in the phloem	Turk and Smeekens (1999); Dueck <i>et al.</i> (1998)
Rice	expression of soybean glycinin	increased vitamin B6-content (+50%)	Momma <i>et al.</i> (1999)
Rice	expression of provitamin A biosynthetic pathway	formation of unexpected carotenoid derivatives (beta-carotene, lutein, zeaxanthin)	Ye <i>et al.</i> (2000)
Soybean	expression of glyphosphate (EPSPS) resistance	higher lignin content (20%) at normal soil temperatures (20°C); splitting stems and yield reduction (up to 40%) at high soil temperatures (45°C)	Gertz <i>et al.</i> (1999)
Wheat	expression of glucose oxidase	phytotoxicity	Murray <i>et al.</i> (1999)
Wheat	expression of phosphatidyl serine synthase	necrotic lesions	Delhaize <i>et al.</i> (1999)

^aData from publicly available reports.

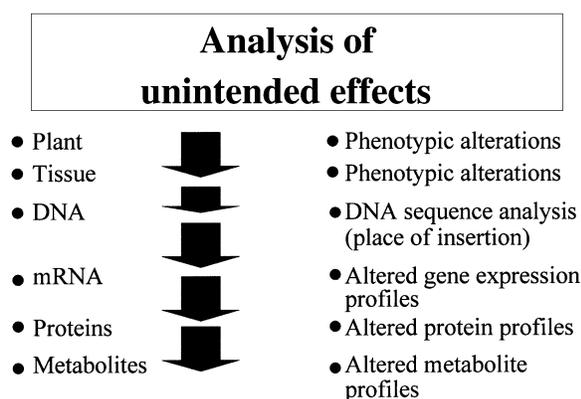


Figure 3. Different integration levels for the detection of unintended effects.

pound-specific) approach, or the non-targeted (profiling/fingerprinting) approach.

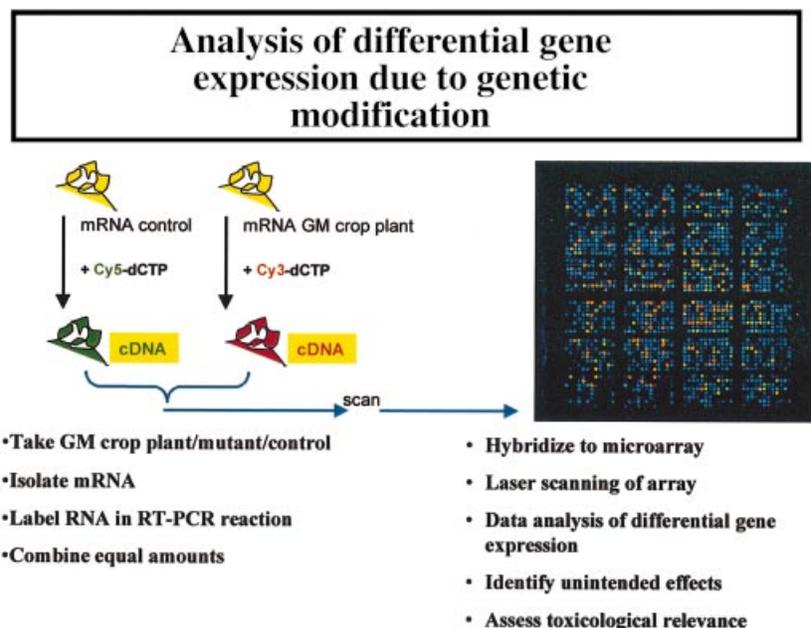
Targeted approach using single compound analysis. For any given transformation event, targeted studies should include baseline analyses of a number of key nutrients such as proteins, carbohydrates, fats, vitamins and other nutritional/anti-nutritional compounds which, if unintentionally modified, might affect nutritional value and safety. Selection of key nutrients and toxicants needs to take into account the target species, structure and function of the inserted gene(s), and possible interferences in metabolic pathways (Figure 3). Selection of compounds may be limited to a restricted number which represents essential biochemical/physiological pathways in the organism. It is

plausible, but not proven, that expected changes in the metabolism as a possible result of the genetic modification will be identified by analysis of a great number of components, but unexpected changes are merely identified by chance. The targeted approach has severe limitations with respect to unknown anti-nutrients and natural toxins, especially in less well known crops.

Non-targeted approach using profiling methods. An alternative (non-targeted) approach for the detection of unintended effects is the use of so-called profiling techniques. New methods are being developed which allow for the screening of potential changes in the physiology of the modified host organism at different cellular integration levels: at the genome level; during gene expression and protein translation; and at the level of metabolic pathways. Many factors, such as genetic characteristics (cultivar, individual, isogenic lines, heterosis); agronomic factors (soil, fertilizers, plant protection products); environmental influences (location effect, weather, time of day, stress); plant–microbe interactions; maturity stage; and post-harvest effects determine the morphological, agronomic and physiological properties of a food crop. Screening for potential changes in these characteristics in genetically modified plants becomes more important as the newer genetic alterations changing agronomical or nutrition-related properties are more complex, involving insertion of large DNA fragments or clusters of genes.

DNA analysis. Localization and characterization of the place(s) of insertion are the most direct approaches to

Figure 4. The microarray technology is currently used to develop a non-biased system for the detection of altered gene expression in genetically modified crop plant varieties in comparison to the traditional parent line.



predicting and identifying possible occurrence of (un-)intended effects due to transgene insertion in recipient-plant DNA. Data for transgene flanking regions will give leads for further analysis, in the case of a transgene insertion within or in the proximity of an endogenous gene. Transgene chromosomal location and structure can be detected by various methods such as genomic *in situ* hybridization (Iglesias *et al.*, 1997) and fluorescence *in situ* hybridization (Pedersen *et al.*, 1997), and by direct sequencing of flanking DNA (Spertini *et al.*, 1999; Thomas *et al.*, 1998). Knowledge of plant genomes is still limited, including the reliability of annotations in genomic databases, but the understanding of the genomic code and the regulation of gene expression in relation to the networks of metabolic activity is increasing. Therefore, the sequencing of the place of insertion(s) will become increasingly informative.

Gene expression analysis. The DNA microarray technology is a powerful tool to study gene expression. The study of gene expression using microarray technology is based on hybridization of mRNA to a high-density array of immobilized target sequences, each corresponding to a specific gene. mRNAs from samples to be analysed are labelled by incorporation of a fluorescent dye and subsequently hybridized to the array. The fluorescence at each spot on the array is a quantitative measure corresponding to the expression level of the particular gene. The major advantage of the DNA microarray technology over conventional gene profiling techniques is that it allows small-scale analysis of expression of a large number of genes at the same time, in a sensitive and quantitative manner (Schena *et al.*, 1995, 1996). Furthermore, it allows com-

parison of gene-expression profiles under different conditions. The technology and the related field of bioinformatics are still in development, and further improvements can be anticipated (Van Hal *et al.*, 2000).

The potential value of the application of technology for the safety assessment of genetically modified food plants is currently under investigation (E.J.K., unpublished results). The tomato is used as a model crop. To study differences in gene expression, two informative tomato expressed-sequence-tag (EST) libraries are obtained, one consisting of ESTs that are specific for the red stage of ripening, and the other for the green, unripe stage. Both EST libraries are spotted on the array and, in addition, selected functionally identified cDNAs, selected on the basis of their published sequence. The array is subsequently hybridized with mRNAs that are isolated from a number of different genetically modified varieties under investigation, as well as with the parent line and control lines. Preliminary results show that reproducible fluorescence patterns may reveal altered gene expression outside the ranges of natural variation, due to different stages of ripening (Figure 4). Prospects are that this method may effectively be used to screen for altered gene expression and, at the same time, provide initial information on the nature of detected alterations, whether the observed alteration(s) may affect the safety or nutritional value of the food crop under investigation.

Proteomics. Correlation between mRNA expression and protein levels is generally poor, as rates of degradation of individual mRNAs and proteins differ (Gygi *et al.*, 1999). Therefore, understanding of the biological complexities in

the plant cell can be expanded by exploiting proteomics, a technique that analyses many proteins simultaneously and will contribute to our understanding of gene function. Particularly, recent developments in mass spectrometry have increased the applicability of two-dimensional gel electrophoresis in the studies of complex protein mixtures. Proteomics can be divided into three main areas: (i) identification of proteins and their post-translational modifications; (ii) 'differential display' proteomics for quantification of the variation in contents; and (iii) studies of protein-protein interactions.

The method most often used for analysing differences in protein pattern is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by excision of protein spots from the gel, digestion into fragments by specific proteases, and subsequently analysis by mass spectrometry (peptide mass fingerprinting). It allows the identification of proteins by comparing the mass of peptide fragments with data predicted by genetic or protein sequence information. Other much faster technologies, such as protein chip-based (microarray) approaches, are under development (MacBeath and Schreiber, 2000; Pandey and Mann, 2000). In addition, major technical hurdles remain to be overcome: proteins may constantly change in their secondary, tertiary and quaternary structures, depending on transfer and expression in different tissues and cellular compartments, which may profoundly influence their electrophoretic behaviour and molecular mass.

When searching for unintended changes by 2-D PAGE, the first step is to compare proteomes of the lines under investigation. If differences in protein profiles are detected, normal variations should be evaluated. If the profiles are outside normal variations, identification of the protein must be carried out, which may lead to further toxicological studies. Moreover, metabolic changes may be looked at if the identified protein has a known enzymatic activity.

There is one example of the use of proteomics to study alterations in the composition of a genetically modified plant, which illustrates that a targeted change in the level of a specific protein can result in other proteins being affected. The improvement in rice storage proteins by antisense technology resulting in low-glutelin genetically modified rice for commercial brewing of sake has been associated with an unintended increase in the levels of prolamins (FAO/WHO, 2000b). This would not have been detected by standard analyses such as total protein and amino acid profiling, but was observed only following SDS-PAGE.

Machuka and Okeola (2000) used 2-D PAGE for the identification of African yam bean seed proteins. Prominently resolved polypeptide bands showed sequence homology with a number of known anti-nutrient

and inhibitory proteins, which may have implications for the safe use of these seeds as human food.

Chemical fingerprinting. A multi-compositional analysis of biologically active compounds in plants – nutrients, anti-nutritional factors, toxicants and other relevant compounds (the so-called metabolome) – may indicate whether intended and/or unintended effects have taken place as a result of genetic modification. The three most important techniques that have emerged are gas chromatography (GC), high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). These methods are capable of detecting, resolving and quantifying a wide range of compounds in a single sample. For instance, metabolic profiling of isoprenoids by an HPLC method was described recently with applications to genetically modified tomato and *Arabidopsis* (Fraser *et al.*, 2000). The potential of GC as a metabolic profiling method for plants was demonstrated some 10 years ago (Sauter *et al.*, 1991), and GC/MS has been established as the most versatile and sensitive profiling method in the past 2 years following its systematic development by Roessner *et al.* (2000); Fiehn *et al.* (2000a); Fiehn *et al.* (2000b). Recently, it has been shown that the use of chemical fingerprinting techniques such as off-line LC-NMR may provide information on possible changes in plant matrices due to variations in environmental conditions (Lommen *et al.*, 1998). Determination of a chemical fingerprint was based on the detection of alterations in ¹H-NMR spectra obtained from different water and organic solvent extracts from genetically modified tomato varieties, such as the antisense RNA exogalactanase fruit, and from their non-modified counterpart(s) (Noteborn, 1998; Noteborn *et al.*, 1998; Noteborn *et al.*, 2000). Differences in concentration of low molecular weight components (MW < 10 kDa) could be traced by subtraction of the ¹H-NMR spectra.

Application of these techniques will provide more detailed information on possible changes than can be obtained from single-compound analysis. Once differences have been identified, further safety evaluation of the observed differences may be needed by specific *in vitro* and/or *in vivo* testing. The design of such experiments will focus on the differences observed with the profiling methods. However, a number of problems must be addressed before such methods can be used on a routine basis: (i) standardization of sample collection, preparation and extraction procedures; (ii) standardization and validation of measurements; (iii) limited availability of data on profiles and natural variations; and (iv) lack of bioinformatic systems to treat large data sets.

Currently, different methods are tested for the detection and characterization of unintended effects as a result of genetic modification. Within an EU project, GMOCARE (QLK1-1999-00765; [© Blackwell Science Ltd, *The Plant Journal*, \(2001\), 27, 503–528](http://www.rikilt.wagenin-</p>
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Table 7. Antibiotic- and herbicide-resistance genes commonly present in commercial- and field-tested genetically modified crops^a

Gene	Gene product	Antibiotic	Gene source
<i>nptII</i>	neomycin phosphotransferase II	kanamycin, neomycin, geneticin (G418), paromomycin, amikacin	<i>Escherichia coli</i> , transposon Tn5
<i>bar</i>	phosphinothricin acetyltransferase	glufosinate, L-phosphinothricin, bialaphos	<i>Streptomyces hygroscopicus</i>
<i>pat</i>	phosphinothricin acetyltransferase	glufosinate, L-phosphinothricin, bialaphos	<i>Streptomyces viridochromogenes</i>
<i>bla</i>	beta-lactamase	penicillin, ampicillin	<i>Escherichia coli</i>
<i>aadA</i>	aminoglycoside-3'-adenyltransferase	streptomycin, spectinomycin	<i>Shigella flexneri</i>
<i>hpt</i>	hygromycin phosphotransferase	hygromycin B	<i>Escherichia coli</i>
<i>nptIII</i>	neomycin phosphotransferase III	amikacin, kanamycin, neomycin, geneticin (G418), paromomycin	<i>Streptococcus faecalis</i> R plasmid
<i>cp4 epsps</i>	5-enolpyruvate shikimate-3-phosphate synthase	glyphosate	<i>Agrobacterium</i> CP4
<i>epsps</i>	5-enolpyruvate shikimate-3-phosphate synthase	glyphosate	<i>Zea mays</i> , <i>Petunia hybrida</i> , <i>Arabidopsis thaliana</i>
<i>gox</i>	glyphosate oxidoreductase	glyphosate	<i>Achromobacter</i> LBAA
<i>bxn</i>	bromoxynil nitrilase	bromoxynil	<i>Klebsiella pneumoniae</i> var. <i>ozaenae</i>
<i>als</i>	acetolactate synthase	sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidylbenzoates	<i>Arabidopsis thaliana</i> , <i>Nicotiana tabacum</i> , <i>Brassica napus</i>

^aData from Metz and Nap (1997) (except *bla*).

gen-ur.nl/euprojects/euprojects.html), the above-mentioned approaches are exploited, including functional genomics, proteomics and metabolite profiling.

Assessment of marker genes

The most commonly used marker genes are those that code for resistance to herbicides or antibiotics (Table 7). The use of herbicide-resistant genes can be twofold: for selection purposes; and/or for altering the agronomic characteristics of a plant. In particular, the use of antibiotic-resistance genes is subject to controversy and intense debate, because of the risk of transfer and expression in bacteria which could compromise the clinical or veterinary use of certain antibiotics. Risk assessment of selectable marker genes with respect to the consumption by humans and animals of genetically modified foods or feed should focus, as with any new gene transfer, on micro-organisms residing in the gastro-intestinal tract of humans and animals, on the toxicity and allergenicity of newly expressed proteins, and on the impact of horizontal gene transfer. Health aspects of marker genes have been dealt with by, among others, WHO (1993); the Nordic Council (Karenlampi, 1996); FAO/WHO (1996); FAO/WHO (2000b). There is general agreement that transfer of antibiotic resistance genes from plants to micro-organisms residing in the human gastro-intestinal tract is unlikely to occur, given the complexity of steps required for gene transfer, expression, and impact on antibiotic efficacy (FAO/WHO, 1996). Under conditions of selective pressure (i.e. oral therapeutic use of the corresponding antibiotic), a select-

able marker may provide selective advantage to the recipient micro-organism.

Transfer of plant DNA to microbial or mammalian cells would require the following steps (FAO/WHO, 2000b): release of specific genes in the plant DNA; survival of the gene(s) under gastro-intestinal conditions (plant, bacterial, mammalian nucleases); competitive uptake of the gene(s); recipient bacteria or mammalian cells must be competent for transformation, and gene(s) must survive restriction enzymes; insertion of the gene(s) into the host DNA by rare repair or recombination events.

There are no data available indicating that marker genes in genetically modified plants transfer to microbial or mammalian cells. Transfer and expression of plant genes in bacteria have been observed under laboratory conditions, and only when homologous recombination was possible (Nielsen *et al.*, 1997). This would imply that an antibiotic resistance-marker gene is introduced from plants into bacteria only if the same gene or other genes with identical sequences were present in the bacteria. Model experiments with mice indicated the transfer of bacterially derived DNA fragments into mouse cells (Schubbert *et al.*, 1998). These results have been criticized, along with others, regarding possible artefacts created during the analysis of foreign insertions in leukocyte DNA (Beever and Kemp, 2000). A relevant consideration for the assessment of horizontal gene transfer, if it occurs, is the consequences of the transfer. Information must be available on the role of the antibiotic in human and veterinary

use, its specific therapeutic spectrum, existing resistance levels in the environment, and possible alternatives for treatment of diseases.

The 2000 FAO/WHO Consultation concluded: 'For certain antibiotic resistance genes currently in use in genetically modified plants, available data suggest that consequences of horizontal gene transfer will be unlikely to pose a significant threat to the current therapeutic use of the respective drugs. With other genes that confer resistance to drugs that are important in specific medical use, or to drugs that have limited alternative therapies, the possibility of transfer and expression of these genes is a risk that warrants their avoidance in the genomes of widely disseminated GMOs and foods and food ingredients' (FAO/WHO, 2000b). It then goes on: 'In future developments, the Consultation encourages the use of alternative transformation technologies, if available and demonstrated to be safe, that do not result in antibiotic resistance genes in genetically modified foods. If further development of alternative technologies is required, additional research should be strongly encouraged'.

Non-antibiotic (alternative) marker genes such as tryptophane decarboxylase, β -glucuronidase and xylulose/phosphomannose isomerase should be evaluated according to the characteristics of the newly encoded proteins and metabolites formed as result of enzymatic reactions. Furthermore, the risks of the presence of multiple markers and of multiple copies of markers should be evaluated. In one example, the isopentenyl transferase (*ipt*) gene for plant hormone production (cytokines) allows modified cells to form shoots when cultured in dexamethasone-enriched media after the modification event (Kunkel *et al.*, 1999). Another way is to use the *xyIA* gene, which encodes xylose isomerase, enabling the genetically modified plant cell to grow in cultures with the sugar xylose added, which is normally toxic to the plant cells. Novartis, for example, has commercialized the *manA* gene as 'Positech', which encodes phosphomannose isomerase, that allows plant cells to be sustained in media containing mannose-6-phosphate (Joersbo *et al.*, 1998).

In addition, methods have been developed to excise genes after successful introduction, such as the CreLox system in which Cre is an enzyme that removes the stretch of DNA flanked by the Lox sequences (Gleave *et al.*, 1999). In a recent version of the CreLox system, both the antibiotic selection marker and the Cre recombinase gene were contained between the Lox sequences of the vector DNA that was introduced into plants. After successful transformation, expression of the recombinase gene was induced, and the marker and recombinase genes were subsequently removed by the recombinase (Zuo *et al.*, 2001).

New models for safety testing, detection of unintended effects, gene transfer, detection and traceability of genet-

ically modified foods are currently under development in the EU-funded research and technology development (RDT) projects SAFOTEST (QLK1-1999-00651); GIOCARE (QLK1-1999-00765); GMOBILITY (QLK1-1999-00527); and Qpcrgmofood (QLK1-1999-01301), clustered in the Thematic Network ENTRANSFOOD (<http://www.entransfood.nl>).

Post-marketing surveillance

In its guidelines for the food-safety evaluation of GMOs, the EU Scientific Committee on Food states that long- and short-term effects of eating novel foods can be (further) assessed by nutritional and safety post-market surveillance (PMS) (EU, 1997b). The Joint FAO/WHO Expert Consultation on Foods derived from Biotechnology (FAO/WHO, 2000b) also advocated monitoring of changes in nutrient levels in novel foods, and evaluation of their potential effect on nutritional and health status. Current practice in the pharmaceutical sector cannot be used as a model for PMS in the food sector, as the physician or other medical professional usually plays a crucial role in the collection of data on adverse effects of new pharmaceutical products. In addition, pharmaceutical products are separately packaged, usually taken by the patient during a limited time-frame, and patients will in many cases already be prepared for some adverse side-effects of the medication. These factors will enable adverse effects to be linked more easily to ingested medicines than to food products or ingredients. Different strategies for post-marketing surveillance in the food-producing sector are available for food products that can easily be traced and identified. These methods vary from direct consumer feedback to the repurchase of products to determine the quality of the product on the shelf.

These PMS strategies for food products will, in most cases, not be directly applicable to GMO-derived food products. Most products derived from a genetically modified plant will be used in products with slight changes in the recipes, depending on the genetically modified plants (varieties) that are available to the producer. As these changes will, in most cases, not be reflected on the label, it will be difficult (or impossible) for the consumer to relate adverse effects to the specific ingredient or GMO component of an ingredient. Only in the case of a genetically modified plant with an added value that the producer would like to communicate to the consumer, an identity-preserved food-production chain with constant composition (control) and clear labelling may enable the consumer to trace any adverse effects back to the product. Adequate GMO detection and identification methods, in combination with repurchasing strategies, may supply comparable information on complex food products possibly containing specific genetically modified varieties, but

this approach will be too elaborate and costly for the routine application that is necessary to be meaningful. Only acute effects that are associated with high intakes of a substance are likely to become visible by PMS as, in general, long-term and/or rare effects usually require targeted epidemiological techniques beyond any normal post-marketing data collection. This will be even more valid for GMO-derived products outside identity-preserved food-production chains. As an example, all consumers who have reported an allergenic reaction after the consumption of maize products that (may have) contained ingredients derived from the unapproved StarLink maize variety did not report this until after the publication of the unintentional entry of this variety into specific food-product chains. It is questionable whether these adverse effects would ever have been reported if the media had not paid so much attention to the affair. We know very little of the potential long-term effects of any food, and many chronic health effects are multifactorial.

The British Food Standards Agency began a feasibility study in 1999 to determine whether long-term monitoring of novel foods is possible (Baynton, 1999). The study aims to obtain data on household consumption patterns and supermarket sales in the 239 local authority districts in Great Britain. The idea is that if variation at district level regarding food purchasing and consumption can be detected, it may be possible to link this variation to health outcomes at district level. The results of the study will lead to recommendations with respect to the future surveillance of novel foods.

Some cases of PMS in relation to food constituents have already been documented. Examples include the food additive aspartame, a high-intensity sweetener; and Olestra, a fat substitute used in snack foods. In the case of aspartame, the primary goal was to obtain more reliable information on the actual intake of the additive in comparison to pre-marketing projections. In the PMS, 5000 individuals in more than 2000 households per year were surveyed for a 14-day period from 1984–92. The study concluded that the consumption of aspartame was well below the ADI. Another conclusion of this study was that medical passive surveillance systems (spontaneous reports of adverse health effects) may be useful for identifying infrequent negative effects of a food additive, but when a food additive gains widespread use, the usefulness of this approach will significantly diminish (Butchko *et al.*, 1994).

In the case of Olestra, it was investigated whether the consumption of Olestra-containing snack foods might affect nutritional status, especially in relation to the serum concentrations of different carotenoids and fat-soluble vitamins, as experimental studies had shown that the uptake of fat-soluble nutrients in the gut may be affected by Olestra. For this study, 403 Olestra-consuming

adults were selected. No such adverse effects were found; however, it is advocated that monitoring of Olestra consumption and its effects on nutritional status should be continued, in particular when additional new food products containing Olestra come on to the market (Thornquist *et al.*, 2000). Another post-marketing study (Cooper *et al.*, 2000) on the same fat substitute was performed by, among other methods, determining the macular pigment optical density in 280 individuals, both Olestra consumers and colleagues, as a measure for the yellow carotenoid pigments lutein and zeaxanthin in the central area of the retina. No significant associations were reported here either. From these examples, it can be concluded that PMS may be informative in those cases where clear-cut questions are the basis for the surveillance.

Very important in the discussion of PMS in relation to the evaluation of GMO-derived products is the consideration that novel food products should not be placed on the market if any question associated with negative health effects is left unanswered during the pre-market assessment. Questions in relation to (unpredictable) allergenicity and alterations in the nutritional status of consumers as a result of the marketing of a particular novel food may be answered by PMS. A major challenge, however, will be to set up informative PMS systems for products that have not been monitored or surveyed so far, which will be relatively difficult to trace, and will participate in different food-production chains on a variable basis.

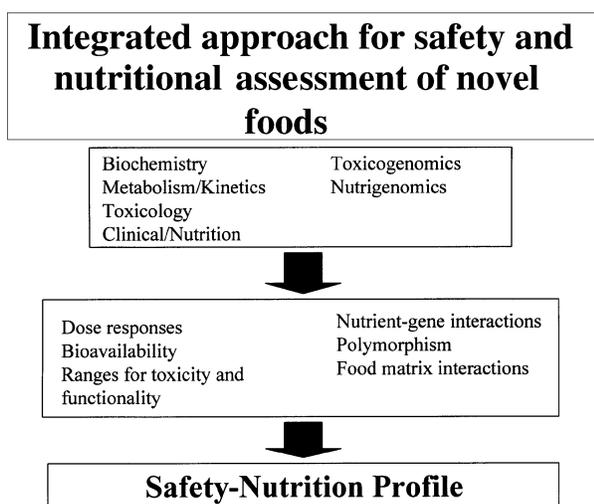
Future developments

A number of genetically modified plants and foods obtained through extensive genetic modification(s) with the purpose of improving agronomic or food-quality traits (Table 8) will soon enter the commercial market. These developments are reviewed in more detail elsewhere (Kleter *et al.*, 2000).

With respect to safety assessment, these new (second-generation) products should, in principle, also be assessed applying the concept of substantial equivalence. The recipient species in many cases provides a relevant baseline for the safety evaluation. For instance 'golden rice', with the β -carotene biosynthesis pathway introduced into the endosperm, contains genes from *Narcissus pseudonarcissus* coding for phytoene synthase and lycopene-cyclase under control of the rice glutelin promoter, as well as a bacterial gene from *Erwinia* coding for phytoene-desaturase under the 35S promoter (Ye *et al.*, 2000). β -Carotene was predominantly present, followed by the unexpected presence of the xanthophylls lutein and zeaxanthin. Non-modified isogenic rice functions as a comparator to identify potential changes in the composition, which should then be further assessed. The extent

Table 8. Examples of novel food crops under development

Crop	Trait	Reference
Canola	increased vitamin E	Shintani and Della Penna (1998)
Coffee bean	caffeine free	Stiles <i>et al.</i> (1998)
Papaya	adapted to aluminium-rich soils	De la Fuente <i>et al.</i> (1997)
Potato	less darkening on bruising	Coetzer <i>et al.</i> (2001)
Rice	introduced beta-carotene	Ye <i>et al.</i> (2000)
Rice	increased iron	Goto <i>et al.</i> (1999); Potrykus <i>et al.</i> (1999)
Rice	decreased allergenicity	Nakamura and Matsuda (1996); Tada <i>et al.</i> (1996)

**Figure 5.** Integrated approach for safety evaluation of genetically modified foods.

and target of the genetic modification, and the resulting alterations in metabolic pathways in the modified organism, guide the safety assessment and may lead to a more extensive toxicological safety evaluation compared to the genetically modified products that are now commercially available (OECD, 2001b).

The assessment of genetically modified plants/foods with enhanced nutritional properties should focus on the simultaneous characterization of inherent toxicological risks and nutritional benefits. This requires an integrated, multidisciplinary approach, incorporating molecular biology, toxicology, nutrition and genetics (Figure 5). Issues to be addressed are: (i) evidence for nutritional/health claims and target population(s); (ii) toxicological and beneficial dose ranges of selected compounds; (iii) impact on overall dietary intake and associated effects on consumers; (iv) interactions between food constituents and food matrix effects; and (v) possibilities for effective post-market surveillance, if necessary. Assessment of the safety of this type of foods is the crucial part of the evaluation,

regardless of the potential benign effects of certain food constituents.

Classical toxicological, nutritional and kinetic studies may answer some of the questions related to safety and nutritional margins, in parallel with animal-feeding trials with whole foods/feeds, taking the limitations of this type of studies into account. But new innovative techniques such as the DNA microarray technology and proteomics are needed in order to characterize the complex interactions of bioactive food components at the molecular and cellular levels. Large-scale screening of the simultaneous expression of a large number of genes and synthesized proteins will provide relevant information concerning the complex relationships between human/animal exposure to bioactive food constituents and their specific effects. Moreover, insight can be gained in individual variabilities in biological responses (polymorphism), as well as in food-matrix oriented interactions.

Safety assessment of genetically modified food crops different from that of conventional crops?

Whenever changes are made in the way of food production or processing, or when new foods without a history of use enter the market, a full safety and nutritional assessment with respect to implications for the consumers should be made. Various regulations have defined categories of foods and new food-processing methods which require such a safety assessment (see above).

The safety assessment of conventional crops is primarily based on analysis of agronomic performance and a by definition-limited analysis of known macro- and micronutrients, anti-nutrients and toxicants. Products with an unusual agronomic performance, taste, or harmful levels of specific compounds are rejected from the traditional breeding programme, for example, potato with high glycoalkaloid content (Harvey *et al.*, 1985), squash and zucchini containing cucurbitacin E (Coulston and Kolbye, 1990), and celery containing furanocoumarins (Beier, 1990). A long history of traditional breeding has given insight into the presence of nutritionally beneficial com-

pounds and of anti-nutrients and toxicants in food plants, in which levels have been increased and/or diminished, respectively, through extensive breeding. This (targeted) approach has great value and has resulted in a healthy and relatively safe food package, and should still be the leading principle when assessing the safety and wholesomeness of traditionally bred food crops. In the case of new plant varieties developed with traditional techniques with no appropriate comparator or history of safe use, application of the new profiling techniques is of great value for the assessment of the safety of these crops.

Our understanding of the relationship between dietary intake of specific foods/food components and human safety and health increases rapidly, even at the level of individual responses through the development of modern genomic and proteomic techniques. This will, in the near future, guide plant breeders more precisely in developing crops with improved safety and wholesomeness.

Conclusions

Safety assessment of genetically modified foods should be carried out on a case-by-case basis, comparing the properties of the new food with those of a conventional counterpart. This approach, the concept of substantial equivalence, identifies potential differences between the genetically modified food and its counterpart, which should then be further assessed with respect to their safety and nutritional implications for the consumer. The concept as developed by OECD has been endorsed by FAO/WHO, and contributes to an adequate safety assessment strategy. No alternative, equally robust strategy is available.

Application of the concept of substantial equivalence needs further elaboration and international harmonization with respect to selection of critical parameters, requirements for field trials, statistical analysis of data, and data interpretation in the context of natural (baseline) variations.

Testing of whole (genetically modified) foods in laboratory animals has its problems. The specificity and sensitivity of the normally applied methods is usually poor. There is a need for improvement of the test methodology using *in vivo* and *in vitro* models. Moreover, there is a need for standardization and harmonization of methods to test the long-term safety of whole foods.

Present approaches to detecting expected and unexpected changes in the composition of genetically modified food crops are primarily based on measurements of single compounds (targeted approach). In order to increase the possibility of detecting secondary effects due to the genetic modification in plants that have been extensively modified, new profiling methods are of interest and should be further developed and validated (non-targeted

approach). Application of these techniques is of particular interest for genetically modified foods with extensive genetic modifications (gene stacking) meant to improve agronomical and/or nutritional characteristics of the food plant.

Pre-market safety assessment of genetically modified foods must provide sufficient safety assurance. The use of post-marketing surveillance as an instrument to gain additional information on long-term effects of foods or food ingredients, either GMO-derived or traditional, should be further explored, but the requirement of routine application will entail large costs for limited amounts of information, and does therefore not seem desirable. Only in specific cases where, for example, allergenicity of newly introduced proteins cannot be excluded, or when exposure assessment is hampered by insufficient insight into the diets of specific consumer groups, post-marketing surveillance strategies may be employed.

The assessment of genetically modified plants/foods with enhanced nutritional properties should focus on the simultaneous characterization of inherent toxicological risks and nutritional benefits. This requires an integrated multidisciplinary approach, incorporating molecular biology, toxicology, nutrition and genetics. New innovative techniques, such as the DNA microarray technology and proteomics, should be applied in order to characterize the complex interactions of bioactive food components at the molecular cellular level.

Current food safety regulations for traditionally bred food crops are, in practice, less stringent compared to those applied to genetically modified foods. A long history of traditional breeding has given relevant insight into the presence of nutritionally beneficial and adverse compounds, and which levels have been increased or diminished, respectively, through extensive breeding. This (targeted) approach has great value and has resulted in a healthy and relative safe food package, and should still be the leading principle when assessing traditionally bred food crops. In the case of new plant varieties with no appropriate comparator or history of (safe) use, application of the new profiling techniques is of great value for characterization of conventionally bred food crops.

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