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Review

Genetic engineering of crop plants for insect resistance – a critical review

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Abstract

Genetically engineering inherent crop resistance to insect pests offers the potential of a user-friendly, environment-friendly and consumer-friendly method of crop protection to meet the demands of sustainable agriculture in the 21st century. Work to date has concentrated on the introduction of genes for expression of modified *Bacillus thuringiensis* (Bt) toxins. Impressive results on the control of Bt-susceptible pests have been obtained in the laboratory and the field, and the first commercial Bt transgenic crops are now in use. A main alternative approach exploits plant-derived insect control genes. Enhanced resistance to a wide spectrum of pests has been demonstrated in laboratory trials of transgenics expressing various protease inhibitors, lectins, etc. and some promising field trials have been carried out, but the scale of effects produced by plant-derived insect control genes has not been deemed convincing enough to lead to serious attempts at commercialization. Both classes of compounds have limitations: there have been serious failures in resistance to targeted pests in Bt cotton; most plant-derived resistance factors produce chronic rather than acute effects; and many serious pests are simply not susceptible to known resistance factors. We have analysed the characteristics which would be desirable in an ideal transgenic technology: these include being environmentally benign, relatively inexpensive to develop, with a potentially wide spectrum of activity (although targetable at pests and not beneficials), generated by a flexible technology that allows any insect site to be targeted and readily adaptable so that alternatives can be produced as required. We are developing such a technology based on the expression of single-chain antibody genes in crop plants which would be compatible with the likely trends in pesticide discovery using biology-driven target-based methods. The importance of a changed, more socially responsible attitude in this sector is emphasised as is the need for much improved presentation of the benefits and need for responsible deployment of genetically engineered crops.

Keywords: Transgenic crops; Genetic engineering; Insect resistance

1. The problem

There is a continuing, if not growing, need to increase the output of world agriculture if the demands of a rising world population are to be met. The basis for this increase must be improved harvest yields of major crops from existing cultivated land. One practical means of increasing yield would be to preserve more of what is grown from loss to pests, especially insect pests, which are estimated to consume around 14% of total global agricultural output (Oerke et al., 1994). Insects are not only responsible for massive direct losses of productivity as a result of their herbivoury, but also cause massive indirect losses due to their role as vectors for various plant pathogens. These losses occur despite the extensive use of pesticides and fungicides. In the absence of such crop protection measures losses would be much more serious (Table 1).

2. The agrochemical 'solution'

Currently crop protection relies primarily on synthetic chemical pesticides, the basis of a ca. US$10 billion per annum global pesticide market. However, this chemical approach to crop protection is coming under increasing pressure. A good deal of the criticism of the agrochemical industry has an emotive rather than a scientific basis (Taylor, 1994), nevertheless the view is now widely held that such agricultural systems are unsustainable. This

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Table 1
Predicted yield losses in the absence of pesticides of major crops (based on data in Oerke et al., 1994)

Crop	Predicted yield loss (%)
Wheat	52
Rice	83
Maize	59
Potato	74
Soyabean	58
Cotton	84

view is based on their huge costs in terms of non-renewable resources; their inefficiency in terms of the proportion of these resources which actually reach their intended target; the environmentally unacceptable consequences of the preceding criticisms, such as contamination of food chains and water sources and the growing consumer dissatisfaction with the publicly perceived consequences of high input agricultural practices. Total pesticide usage is in decline worldwide, largely due to major reductions in usage in the EU as a result of regulatory and public opinion pressure.

The industries' preferred solutions to this situation tend to be based on risk reduction, rather than use reduction, e.g. the development of more target-specific compounds with less persistence in the environment and the extension of integrated crop management systems (IPM). The tremendous benefits which have accrued to agriculture from the use of synthetic pesticides should not be belittled, but there is clearly an urgent need to develop partial substitution technologies which would allow a much more limited use of synthetic pesticides and yet provide adequate protection of crops within a sustainable agricultural framework, e.g. IPM.

3. Alternatives to agrochemicals

3.1. Biopesticides

Biopesticides, which comprise formulations of the predators, parasitoids and pathogens of pest insects, are becoming more widely used in IPM farming systems. However, they still account for a very small slice (less than 3%) of the pesticide market. The main barrier to their wider use lies in the difficulty of simultaneously managing three different biological populations – predator, prey (pest) and crop; they are particularly difficult to use in annual field crops and so they find their greatest application in glasshouses and forestry where the population dynamics are more readily regulated than in the field.

3.2. Inherent resistance

The importance of host plant inherent resistance or partial resistance as a key component in IPM is increasingly recognised (e.g. van Emden, 1987). The particular advantages of inherent resistance are well known (e.g. Boulter, 1993a) and pest resistance is often now a key characteristic in conventional crop breeding programmes. This may be contrasted with the priorities of breeding programmes in the past which resulted in very few cultivated species retaining the degree of resistance shown by their wild relatives (Feeney, 1976) (e.g. the elimination of gossypols from cotton and glucosinolates in oilseed rape – to be replaced as crop protection agents by sprayed pesticides). The need to manage this inherent resistance is highlighted by recent work (Agrawal, 1998). Plants often synthesise protective chemicals in response to damage by herbivorous insects which later in the plants life can confer an advantage. Apart from using chemicals to enhance the plant's response, there is also the possibility that allowing early season herbivores to feed would give strong resistance to the later season, more economically damaging, pests.

Conventional crop breeding programmes are limited by the need for a source of resistance within the interbreeding gene pool, although there remains considerable scope for further successes, especially from wide crosses. This is one of the constraints which plant genetic engineering can remove, allowing resistance genes from any source (plant, animal, microbial, synthetic) to be introduced into a breeding programme.

3.3. Crop genetic engineering

Genetic engineering of crops offers the prospect of many advantages; not just widening the potential pool of useful genes but also permitting the introduction of a number of different desirable genes at a single event and of reducing the time needed to introgress introduced characters into an elite genetic background.

Since the first reports of transgenic plants appeared in 1984 (Horsch et al., 1984) there has been very rapid progress directed at using this new technology for the practical ends of crop improvement. Protection of crops from insect pests was quickly seized upon as a major goal of plant genetic engineering. The potential size of this market attracted major attention from a number of commercial organisations and the potential economic importance of this sector of biotechnology is finally becoming more widely recognised (Burke and Thomas, 1997).

The practical application of plant genetic engineering involves two equally important technologies; cellular and molecular biology. The list of crop species which are amenable to genetic engineering has grown steadily and now includes all major crop species and many minor,

previously orphan, crops. The list of useful genes for introduction into transgenic crops has not grown at a similar pace, although a number of different genes which might be useful for crop protection have been proposed. In this review, we will concentrate primarily on those genes with demonstrated effects in transgenic plants. By far the greatest research effort in developing pest-resistant transgenic crops has gone into expression of *Bacillus thuringiensis* (Bt) toxins in plants.

3.3.1. Bt toxin

Formulations based on the insect pathogenic bacterium *Bacillus thuringiensis* occupy the position of the worlds leading biopesticide, accounting for ca. 90% of biopesticide sales (Neale, 1997). It has been in limited field use for ca. 40 years for the control of lepidopteran pests. Genes encoding the insecticidal δ-endotoxins were cloned by the early 1980s (Schnepf and Whiteley, 1981) and expression of modified toxin genes in transgenic tobacco and tomato provided the first examples of genetically engineered insect resistance in plants (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987).

The Bt δ-endotoxins constitute a family of related proteins, for which over 140 genes have been described (Crickmore et al., 1998a) and have recently been reclassified into 24 major groups (Crickmore et al., 1998a,b). The different toxins have different specificities for different orders of insects (Lepidoptera, Coleoptera, Diptera) although the susceptibility of different species within a 'susceptible' order varies enormously (Slaney et al., 1992). The (inactive) protoxins are proteolytically cleaved within the insect gut to produce the active 60–65Kd toxin. The activated toxins comprise regions of high homology interspersed with (hyper-) variable regions. The structure / functional roles of the toxins' three domains have been inferred from sequence analysis (Hodgman and Ellar, 1990) and X-ray crystallography (Li et al., 1991).

The mechanism of Bt toxicity has been reviewed by Knowles (1994). Essentially, the active toxin first binds to glycoprotein receptors in the brush border membrane of susceptible insects' midgut epithelium. These receptors obviously play a key role in determining susceptibility/resistance to a particular Bt toxin and their nature is under intensive investigation, with a number of midgut integral membrane glycoproteins, including aminopeptidase and a cadherin-like protein, identified (Knight et al., 1994; Pease et al., 1995; Luo et al., 1996; Yaoi et al., 1997). Following binding, the toxin rapidly and irreversibly inserts into the cell membrane. Insertion results in formation of a pore which leads to epithelial cell lysis. This cytolysis leads to gut paralysis, cessation of feeding and finally (typically after 1–3 days) death from starvation and/or septicaemia.

Although the early transgenic plants containing Bt genes demonstrated some enhanced resistance to target pests it was soon apparent that expression levels from the native bacterial genes were too low to provide adequate protection from major pest species in the field. Substantial increases in expression levels were required and these have been achieved through the use of strong promoters and enhancers and by engineering the codon usage to bring it more into line with plant-preferred codon usage, rather than the A + T rich *Bacillus* preferred usage, and to eliminate undesirable mRNA secondary structure and polyadenylation signals. The result is that the majority of Bt genes now in use for plant genetic engineering have been substantially modified and essentially rebuilt, with expression levels in transgenics very much higher ($100\times$) than was obtainable using native Bt genes (e.g. Perlack et al., 1991; Wong et al., 1992). Bt genes have now been introduced into and expressed in a wide range of crop species, including tobacco and tomato (pub 1987); cotton (1989); rice (1990); potato and brinjal (1992): maize and broccoli (1993); oilseed rape, soyabean and walnut (1994); larch and poplar (1995); sugarcane and apple (1996); peanut, chickpea and alfalfa (1997).

Many field trials of crops expressing these modified Bt genes have been carried out (Table 2) and most of those which have been published have produced some impressive results – e.g. Cry1A-cotton *v* cotton bollworm (*H. zea*) and pink bollworm (*Pectinophora gossypiella*) (Wilson et al., 1992); Cry3A-potato *v* Colorado potato beetle (*Leptinotarsa decemlineata*) (Perlak et al., 1993); Cry1A-elite maize *v* European corn borer (*Ostrinia nubilalis*) (Armstrong et al., 1995).

These transgenic crops have not, however, enjoyed entirely unqualified success in the field. Bt-cotton was reported to have failed to control *H. armigera* in Australia. In 1996, 2 million acres of the US cotton belt (13% of

Table 2
Published field trials of Bt-expressing transgenic crops

Crop	Bt	Target pest	Ref.
Tobacco	Cry1A	Heliothines	Warren et al. (1992)
		H. zea	Hoffmann et al. (1993)
Potato	Cry1A	Tuber moth	Peferoen (1992)
	Cry3A	Colorado potato beetle	Perlak et al. (1993)
Cotton	Cry1A	Pink bollworm	Wilson et al. (1992)
Maize	Cry1A	European cornborer	Koziel et al. (1993) Armstrong et al. (1995)
		H. zea	Sims et al. (1996)
		2° Lepidoptera	Pilcher et al. (1997)
Rice		Stemborers	Fujimoto et al. (1993)
Tomato		Pinworm	Delannay et al. (1989)
Poplar	Cry1A	Gypsy moth	Kleiner et al. (1995)

the total US cotton crop) were planted with Bt-transgenic cotton seed developed by Monsanto. This seed was marketed at a premium price by Delta Pine and Land Co for the control of pink bollworm, tobacco budworm and cotton bollworm. The crop failed to control cotton bollworm on at least 20,000 acres in Texas. Possible causes which have been suggested for this failure include: inadequate expression levels, perhaps influenced by the environment; a naturally resistant local bollworm population, or development of resistance, perhaps due to inadequate resistance management; unusually high population pressure, again perhaps influenced by the environment (Kaiser, 1996). The report requested by the US EPA on the reasons for and consequences of this failure is not yet available to the authors. The following years cotton crop using Monsanto's herbicide resistant transgenics suffered a similar failure (Kleiner, 1997).

Ciba-Geigy's commercial Bt-expressing transgenic maize (targeted at control of European corn borer) has suffered a somewhat different setback in the EU. Imports from the US of grain derived from this transgenic crop were initially approved by the European Commission. However, the (unnecessary) presence of a bacterial promoter/antibiotic (ampicillin) resistance gene and the failure to segregate genetically engineered from conventional grain – which was perceived as denying the consumers the 'right to choose' – resulted in opposition from various national 'approval committees', environmental groups and consumer groups. The end result is a highly confused situation, with several national bans of the grain and a serious setback to public acceptability of transgenic crop products. The confusion is exemplified by the situation in France (a country which produces maize and suffers from cornborer attack) where the transgenic grain is considered safe to eat, but not safe to grow! The importance and complexity of issues of 'acceptability' of transgenic crops has recently been discussed by Boulter (1997).

3.3.1.1. Specificity and durability. The high degree of specificity of Bts is often cited as one of the benefits of their use over synthetic pesticides. However, most crops are not subject to attack by a single pest species but rather by an entire complex of different pests. For example, cotton, although grown under a number of different cropping systems, is subject to losses from a surprising similar pest complex worldwide, principally heliothines, mirids, aphids, spider mites and thrips (Lutterell et al., 1994). Many of these pests are not susceptible to any known Bts. The value of transgenics protected against, e.g. heliothines is likely to be much reduced if chemical pesticides still have to be regularly applied to control, e.g. whitefly. There is a need to identify insect control genes for these currently unsusceptible pests. Because transgene products are essentially confined within the host plant, they are intrinsically specific to those pests which are heinous enough to eat those plants. So, whereas there is a trend in favour of highly selective, narrow spectrum compounds for use as chemical pesticides, with transgenics it may be argued that a broader spectrum of activity is desirable, provided this does not include beneficial insects as well (Hilder and Hamilton, 1994).

Pest insects have shown a remarkable capacity to develop resistance to chemical pesticides with over 500 species of insects now resistant to chemicals (Moberg, 1990). The development of resistance to Bt toxins in laboratory and field populations of pests has set alarm bells ringing in many quarters (McGaughey and Whalon, 1992; Tabashnik, 1994), particularly in view of its position as the premier biopesticide. This situation is particularly acute amongst 'organic farmers', for whom Bt is one of the few acceptable sprays for insect control. Their representatives have argued that whereas their long-term use of Bt sprays has not led to the emergence of resistant insect populations, the widespread use of Bt-transgenics is likely to exert so much selection pressure for resistant insects that this valuable control measure will be lost to them. Various resistance management strategies have been proposed for transgenics, including the use of more than a single resistance factor and the provision of spacial and temporal refugia to ensure survival of susceptible genotypes (e.g. Roush, 1997; Bergvinson et al., 1997; Onstad and Gould, 1998) although there is still no consensus on the optimum strategy (e.g. small versus large refugia, etc.) and further field experience is required. The ability of pests to break down host plant resistance is always a grave risk and ways to delay the onset of insect resistance will be an ongoing debate as transgenic crops are deployed. Although there is insufficient data available to confidently recommend management strategies, it is likely that the durability of transgenic crops will be much higher if they have multi-gene, multi-mechanistic resistance within them (Boulter, 1993b). This provides further incentives to identify novel insect control genes. The technology for introducing and expressing multiple transgenes in crops is available (Hadi et al., 1996; Chen et al., 1998)

The specificity of Bt is such that it was expected to have no direct effects on predator populations (although indirect effects due to sick, suboptimal prey (pests) would be expected). Whilst initial observations tended to support such expectations (e.g. Hoffmann et al., 1992), recent results (Hilbeck et al., 1998) suggest that there may be a reduction in fitness of predatory chrysopid larvae directly attributable in part to preying on Bt-maize fed caterpillars (although there was an unfortunately high control mortality in these experiments). Any direct impact of transgenic Bt on predator populations is likely to be far less marked than would be the case with sprayed insecticides; never-the-less such tritrophic interactions

may be yet another area requiring careful management in the deployment of Bt-transgenic crops.

Of relevance to the durability and specificity of resistance is the question of regulation of expression of transgenes by the use of appropriate promoters. In most cases, insect resistance genes have been inserted with constitutive promoters such as CaMV35S, maize ubiquitin or rice actin 1, which direct expression in most plant tissues. It has been suggested that limiting the time and place of expression by the use of tissue specific (e.g. PHA-L promoter for seed-specific expression or RsS1 promoter for phloem-specific expression) or inducible (e.g. potato *pin*2 wound-induced promoter) promoters might contribute to the management of resistance in the pest and unfavourable interactions with beneficial insects. This tends to be presented as a self-evident truth, rather than a reasoned argument. Of primary importance for pest control is that there is a reliable, effective level of expression of the insect control protein (ICP) in the site on which the pest feeds and at the stage when it is most vulnerable. In most cases these criteria are met by constitutive promoters (note e.g. that phloem expression from CaMV35S matches that from RsS1 in dicots). Constitutive expression also allows broader-spectrum ICPs to be targeted at different components of the pest complex. The greatest risk of resistance build-up would probably arise in the case of prolonged exposure to ineffective levels of the transgene product, a situation which would hardly be tolerated by farmers who in practice would surely adopt additional (different) control measures (deployment of which would in fact reduce the risk of resistance development). As for beneficial insects, the only major ones which actually 'eat' crops are pollinators: note again that CaMV35S notoriously gives very poor/no expression in pollen.

It has also been suggested that restricted expression might minimize any 'yield penalty' associated with transgene expression (e.g. Xu et al., 1993; Schuler et al., 1998). This would become a serious consideration if any such penalty were demonstrated (see later).

There are certain situations where specific promoters would have clear advantages, e.g. for root-feeding cyst nematodes which modify expression at their feeding sites and tend to inactivate general promoters (although even here CaMV35S appears to work (Atkinson, 1993)).

3.3.2. Alternatives to Bt in genetic engineering

The search for alternative ICPs to Bt has concentrated, with a few exceptions, on those derived from plants. In the course of their ca.300 million years of co-evolution (Labandeira and Phillips, 1996), plants have evolved some very effective counter measures to predatory insects – the plants' solutions to the plants' problems. Many different classes of plant proteins have been shown to have some toxic or antimetabolic effect on insects in artificial diet or in vitro investigations (see Shewry and Lucas, 1997) and have been proposed as targets for crop genetic engineering (Table 3). We will concentrate on only those examples of ICPs which have been shown to enhance resistance in transgenic plants.

A common feature of many of these ICPs is that their effects tend to be chronic rather than acutely toxic, so their perceived effects on a pest population are usually

Table 3
Spectrum of effectiveness of proposed ICPs against different organisms

ICP	Lepidoptera	Coleoptera	Hemiptera	Orthoptera	Nematodes	Fungi	Mammals
Serine protease inhibitors	+	+	−	+	+	+	+/−
Thiol protease inhibitors	−	+	−		+		−
Lectins							
Mannose sp	+	+	+		+	+	−
NacGlu sp	+	+	+				+
a-amylase inhibitors	+	+	−				+/−
Enzymes							
Lipoxygenase	+		+				
Acyl-hydrolase		+					
Chitinase	+		−			+	−
Ribosome inactivating proteins	+	+				+	+
Bt toxins							
cryI	+	−	−		−	−	−
cry3	−	+	−		−	−	−

much less dramatic than is the case with synthetic chemical pesticides. They rarely produce 100% kill of insects in any realistic trial, tending rather to increase mortality to a limited extent but to significantly retard insect growth and development. This has been presented as a serious shortcoming by some commentators (Estruch et al., 1997). But such arguments are based on transgenics being used in systems where crop protection relies exclusively on the transgene product totally replacing other protection measures. Plant-derived ICPs attack different targets to synthetic chemicals and may be used in combination with them. Many worthwhile IPM practices, such as the use of short-season varieties, partial resistant varieties and conservation of predators, are aimed at preventing the build up of pest populations to catastrophic levels rather than the total elimination of the pest. Retardation of insect development, leading to a slower rate of population build up, and the reduced fitness of surviving pests should mean that even in situations where transgene expression did not keep the pest population below the threshold for intervention, it should allow a much wider window within which intervention can be successfully employed. This might encourage a greater confidence in the IPM approach on the part of farmers who normally prefer the 'total kill' solution of chemical pesticides, so extension programmes are essential.

Another common feature of many of the plant-derived ICPs is that they tend to have an effect against a rather broad spectrum of pests, as can be seen from Table 3. The possible advantages of a selective broad-spectrum activity of transgene products were discussed above.

3.3.2.1. Protease inhibitors. Disruption of a pests essential amino-acid metabolism by inhibition of protein digestion has been a key target, reviewed by Hilder et al. (1992). Many insects, particularly members of the Lepidoptera, depend on serine proteases (trypsin-, chymotrypsin- and elastase-like endoproteases) as their primary protein digestive enzymes and genes encoding members of various different serine protease inhibitor (SPI) families have been cloned and introduced into transgenic plants. Insects themselves also produce SPIs which are active against, and presumably are involved in regulating, their own digestive proteases. It has been suggested that these could be turned against the insects by expressing them in transgenic plants (e.g. Wasmann et al., 1994; Thomas et al., 1995a, b): an interesting suggestion since these inhibitors have probably evolved specifically to be very effective against the insect proteases. Other pests rely on thiol proteases (cysteine proteases) rather than serine proteases as their primary digestive protease. These have been targeted with thiol protease inhibitors (TPIs). TPIs have been shown to exert chronic effects on particularly important pests such as corn rootworms (*Diabrotica* spp.) against which there are no effective Bts (Edmonds et al., 1996), and cyst nematodes. Nematodes are pests of major economic importance worldwide (we will take the licence of considering nematodes to be legless 'insects' for the purposes of this review). They are a major constraint on tropical agriculture for which few acceptable options for control are currently available.

The first example of the use of a plant-derived ICP gene in transgenic plants came with the constitutive expression (from the CaMV35S gene promoter) of a trypsin inhibitor gene derived from cowpea (*Vigna unguiculata*) in tobacco (Hilder et al., 1987). The results with this gene are typical of those obtained by this approach. Bioassays against tobacco budworm (*H. virescens*) caterpillars showed that transgenics expressing CpTI to ca. 1% caused increased mortality, reduced growth and reduced plant damage. Similar results were subsequently obtained in a field trial in the US against corn earworm (*H. zea*) (Hoffmann et al., 1992). The transgenic tobacco has been shown to have enhanced protection against a range of other lepidopteran pests with similar chronic effects (Table 4).

This, and various other protease inhibitor gene constructs have been introduced into a variety of different transgenic plants, with the results summarised in Table 5. A 'positive effect' indicated in Table 5 means a statistically significant better performance than controls in the bioassays – generally in line with the levels of chronic effects seen in Table 4. 'Statistical significance' is not the same, however, as 'economic importance' and the scale of the effects observed has not been deemed sufficiently convincing to lead to serious attempts at commercialization of these genes.

The complexity of PI/insect interactions is well illustrated in sweetpotato. Sweetpotatoes naturally have high levels of SPIs, yet introduction of an additional, heterologous inhibitor enhances resistance to a sweetpotato pest, *Euscepes postfaciatus* (Golmirizaie et al., 1997). Conversely, expression of a gene encoding a sweetpotato trypsin inhibitor in transgenic tobacco (at a relatively low level for a plant-derived ICP – 0.2%) results in severe growth retardation of *Spodoptera litura* caterpillars fed on it (Yeh et al., 1997). Pests are necessarily adapted to counter the defensive measures of their hosts, but may

Table 4
Effect of CpTI-expressing transgenic tobacco on various different insect species. Figures are % of controls

Insect	Survival	Insect biomass	Plant damage
H. virescens	65	27	40
H. zea (Laboratory)	60	64	34
H. zea (Field)	41	50	48
S. littoralis	69	63	49
M. sexta	55	18	44

Table 5
Published bioassays of transgenic plants expressing introduced protease inhibitor genes

Inhibitor	Plant	Insect[a]	Effect[b]	Reference
Plant derived				
Cowpea-TI	Tobacco	*H. virescens* (Lep)	+	Hilder et al. (1987)
		H. zea (Lep)	+ (Field)	Hoffmann et al. (1992)
	Potato	*G. pallida* (Nem)	+	Atkinson (1993)
		M. incognita (Nem)	+	Atkinson (1993)
		L. oleracae (Lep)	+	Gatehouse et al. (1997)
	Strawberry	*O. sulcatus* (Col)	+ (Field)	Graham et al. (1996)
	Cabbage	*P. rapae* (Lep)	+	Hao and Ao (1997)
		H. armigera (Lep)	+	Hao and Ao (1997)
	Sweetpotato	*E. postfaciatus* (Col)	+	Golmirizae et al. (1997)
	Rice	*C. suppressalis* (Lep)	+ (Field)	Xu et al. (1996)
		S. infestans (Lep)	+ (Field)	Xu et al. (1996)
Sweetpotato-TI	Tobacco	*S. litura* (Lep)	+	Yeh et al. (1997)
Potato I (CI)	Tobacco	*S. litura* (Lep)	−	McManus et al. (1994)
		T. orichlea (Lep)	−	McManus et al. (1994)
		C. eriosoma (Lep)	+	McManus et al. (1994)
Potato II (T/C-I)	Tobacco	*M. sexta* (Lep)	+	Johnson et al. (1989)
		S. exigua (Lep)	−	Jongsma et al. (1995)
	Rice	*S. inferens* (Lep)	+ (Field)	Duan et al. (1996)
	Poplar	*P. versicolor* (Col)	+	Klopfenstein et al. (1997)
Tomato I (CI)	Tobacco	*M. sexta* (Lep)	−	Johnson et al. (1989)
Tomato II(T/C-I)	Tobacco	*M. sexta* (Lep)	+	Johnson et al. (1989)
Soyabean KTI	Tobacco	*H. virescens* (Lep)	+	Gatehouse et al. (1993)
Rice OZC-1	Poplar	*C. tremulae* (Col)	+	Leple et al. (1995)
	Tobacco	*G. pallida* (Nem)	+	Urwin et al. (1995)
Insect-derived				
Manduca serpin	Tobacco	*B. tabaci* (Hom)	+	Thomas et al. (1995a)
	Cotton	*B. tabaci* (Hom)	+	Thomas et al. 1995b
Manduca E-I	Alfalfa	Thrips (Thy)	+	Wasmann et al. (1994)

[a]Parentheses indicate insect order, Col = Coleoptera; Hom = Homoptera; Lep = Lepdoptera; Thy = Thysanoptera; or the Phylum Nematoda (Nem).
[b] + indicates a significant difference between transgenics and controls; − = no significant difference.

still be susceptible to a related defence mechanism from a non-host plant. Polyphagous pests are likely to be able to adapt more readily than specialist feeders. Deployment of PIs in an insect control strategy clearly requires detailed analysis of any particular pest/crop situation. The range of dissociation constants (K_d) for different PIs with specific proteases is huge and Christeller and Shaw (1989) have proposed that K_d might be used to select the most effective inhibitor for gene transfer in any particular situation. In line with this suggestion is the significantly better performance of transgenic tobacco expressing high levels of Kunitz trypsin inhibitor from soya bean (SBTI) compared with CpTI-tobacco in bioassays against *H. virescens*, in which species proteolysis by gut extracts was at least 40 fold more susceptible to inhibition by SBTI than CpTI (Gatehouse et al., 1993). CpTI was considered, however, to be a particularly useful SPI gene for transfer since, unlike many SPIs, it is not deleterious (at least in the short term) to mammals (Puzstai et al., 1992). Also, although many SPIs are toxic to beneficial insects such as honey bees (*Apis mellifera*) (Malone et al., 1995; Burgess et al., 1996), CpTI is not.

Jongsma et al. (1995) provided the important result that only 18% of the proteinase activity of gut extracts from caterpillars raised on the transgenic plants expressing potato inhibitor II was sensitive to inhibition by inhibitor II, whereas 78% was sensitive in caterpillars reared on control plants. They demonstrated that increased activity of an inhibitor II insensitive protease almost completely compensated for the decline in inhibitor II sensitive activity. The ability of some species to compensate for protease inhibition by switching to an alternative proteolytic activity or up-regulating (overproducing) the existing activity would limit the applicability of the protease inhibitor approach in such species.

3.3.2.2. Alpha-amylase inhibitors. In much the same way that pests protein metabolism has been targeted with genes encoding protease inhibitors, so their carbohydrate metabolism has been targeted with α-amylase inhibitors.

The best characterised α-amylase inhibitors are those from wheat (WAAI) and common bean (*Phaseolus vulgaris*) (BAAI). A preliminary report suggested that expression of WAAI in transgenic tobacco increased mortality of lepidopteran larvae fed on it by 30–40% (Carbonero et al., 1993). This has, however, not been substantiated and other laboratories have experienced difficulties in even obtaining demonstrable expression of WAAI genes in transgenic plants.

The gene encoding BAAI has been expressed in transgenic pea seeds, driven by the *pha*1 gene promoter to direct high levels of expression in seeds, where it did enhance resistance to two species of bean weevil (*Callosobruchus* spp.) which are important storage pests of legume seeds (Shade et al., 1994; Schroeder et al., 1995). Similar enhanced resistance to bean weevils has been demonstrated in the seeds of transgenic adzuki beans (Ishimoto et al., 1996).

3.3.2.3. Lectins. Plant lectins constitute a heterogenous group of sugar-binding proteins which are believed to have protective functions against a wide range of organisms. Feeding trials with purified proteins have shown lectins having various sugar-binding specificities to have chronic effects on survival and/or development of insect pests belonging to several insect orders (e.g. Shukle and Murdock, 1983; Czapla and Lang, 1990; Habibi et al., 1992; Powell et al., 1993).

The first demonstration of enhanced resistance of transgenic plants expressing a foreign lectin used the gene encoding the glucose/mannose-binding lectin from pea (*Pisum sativum*) (Boulter et al., 1990). Bioassays of transgenic tobacco expressing pea lectin against *H. virescens* showed significantly better performance than controls. A key consideration in the decision to work with pea lectin was that it, unlike many insecticidal lectins such as wheatgerm agglutinin (WGA) and phaetohaemagluttinin (PHA), is of low mammalian toxicity. Unfortunately, it is also of rather low insect toxicity.

Greater insecticidal activity is shown by chitin-binding lectins from wheatgerm (WGA) and common bean (PHA). WGA has been expressed in transgenic maize, with the transgenics demonstrating modest inhibition of larval European corn borer and *Diabrotica* (Maddock et al., 1991). However, the mammalian toxicity of this lectin has already been mentioned – it is the opinion of many nutritionalists that lectins such as WGA would be better removed from wheat than introduced into other (food) crops.

A whole new window of opportunity for transgenic crop protection was opened by the demonstration that sap-sucking insects of the order Hemiptera might be controlled by certain lectins (Hilder et al., 1995). A gene encoding the mannose specific lectin from snowdrops (*Galanthus nivalis*: GNA) was introduced into tobacco and transgenics expressing this lectin showed enhanced resistance to the peach potato aphid (*Myzus persicae*) – a serious pest and vector of many virus diseases – with the degree of protection being shown to correlate with level of expression of the introduced lectin. Transgenic potato expressing GNA is similarly partially protected from peach potato aphids (Gatehouse et al., 1996) as well as tuber moth larvae (Gatehouse et al., 1997). These potatoes, however, provided the first suggestion of an adverse tritrophic effect of pest-resistant transgenics, with the claim that the viability of beneficial, predatory ladybirds was reduced when fed on aphids from the engineered plants (see Gledhill and McGrath, 1997). This claim is controversial and, as with Bt (see above), any direct impact of the transgene on predator populations is likely to be far less severe than would result from sprayed insecticides.

3.3.2.4. Enzymes. Transgenic expression of various enzymes have been proposed as crop protection agents. The most obvious candidate is chitinase, since chitin is such an important structural component of insects. Expression of an insect chitinase in transgenic tobacco enhances resistance to some lepidopterans (Ding et al., 1998). We have observed some similar, marginal protective effects from expression of bean chitinase in transgenic tobacco (Gatehouse, 1995). A bacterial endochitinase (from *Serratia marcesens*) has been shown to act synergistically with Bt toxin against *S. littoralis* larvae (Rigev et al., 1996), but not (yet) in transgenic plants.

Cholesterol oxidase secreted by *Streptomyces* has been shown to be acutely toxic to larvae of cotton boll weevil (*Anthonomus grandis*), a particularly difficult pest of cotton (Purcell et al., 1993). A *Streptomyces* cholesterol oxidase gene has been expressed in protoplasts (Corbin et al., 1994: Cho et al., 1995) but insecticidal activity in transgenics has yet to be reported (boll weevils feed exclusively on cotton bolls).

Lipoxygenase has been shown to have some insecticidal effects (Shukle and Murdock, 1983). Although introduced lipoxygenases have been expressed in transgenic plants, enhanced resistance of such plants to insects has not been reported.

3.3.2.5. Alternative microbial compounds. One approach to the discovery of novel insecticidal proteins has been the mass screening of microbial culture supernatants against target pests. Vegetative *Bacillus cereus* culture supernatants have provided two proteins – Vip1 and Vip2 – which together have acute toxic effects on corn rootworms (see Estruch et al., 1997). Some vegetative *B. thuringiensis* culture supernatants provide a protein (Vip3A) which was acutely toxic to *Agrotis* and *Spodoptera* caterpillars (Estruch et al., 1996) The activity of these proteins is very similar to that of Bt δ-endotoxins, although they are clearly distinct from them. Reports of activity in transgenic plants have yet to appear.

3.3.2.6. Predator toxins. Insect predators such as spiders and scorpions produce peptides which are powerful insect neurotoxins. It has been suggested that these might be used to protect transgenic plants and genes encoding some have been introduced into transgenics. The site of action of these neurotoxins is the pests neurendocrine system, usually accessed by injection (either naturally by the predator or experimentally): simple ingestion (as part of a transgenic plant) might not be expected to be a good delivery system to these targets. It has, however, been claimed that expression of a scorpion toxin in transgenic plants results in toxicity to insects fed upon them (Barton and Miller, 1991). Genes encoding neurotoxins from predatory mites (Tomalski and Miller, 1991) and scorpion (Stewart et al., 1991) have been deployed in recombinant baculoviruses where they effectively increase the rate of kill.

3.3.2.7. Pyramiding resistance genes. The effectiveness and durability of resistance in transgenic crops is likely to be greater if they are engineered with multi-gene, multi-mechanistic resistance (Boulter, 1993b). The first example of 'pyramiding' such different resistance to be described involved sexual crossing of transgenic tobacco which expressed the cowpea trypsin inhibitor with transgenic tobacco hemizygous for the pea lectin. F1 progeny expressing neither, either one, or both of the foreign genes were identified. These plants were tested in feeding assays against *H. virescens* larvae. Both genes individually had some protective effect and this effect was precisely additive in the double expressing plants (Boulter et al., 1990)

In order for pyramiding to be successful, it is essential that the mechanisms of resistance are compatible. Introduction of an SPI and the snowdrop lectin into the same transgenic tobacco does not result in additivity of protective effect. Since one of the effects of GNA is believed to be anti-feedant (Powell et al., 1995), it presumably reduces the intake level of the SPI to below the threshold for the latter to have any effect.

MacIntosh et al. (1990) reported that low levels of SPIs enhanced the insecticidal activity of Cry1A, Cry3 and Cry4 against their respective target insects. This effect occurred at levels of inhibitor activity far below those needed for direct effects on the insects. A synthetic sequence encoding the 29 amino-acid squash (*Cucurbita maxima*) trypsin inhibitor (CMTI) was fused to a truncated *cry1Ac* Bt gene and introduced into tobacco. Larval growth reduction bioassays against *H. virescens* showed a ca. 6-fold increase in Bt activity in leaf extracts from transgenics expressing the CMTI-Bt fusion protein compared with transgenics expressing the truncated Bt alone. The mechanism by which such potentiation of Bt activity could occur is unknown. A number of other laboratories have failed to confirm potentiation of Bt by SPIs (Tabashnik et al., 1992). For example, spraying CpTI-expressing transgenic tobacco (Hilder et al., 1987) with sub-optimal concentrations of commercial Bt insecticide resulted in no greater insecticidal effect than either component alone.

Had the above experiment yielded a positive result it would have served to illustrate a novel approach to the use of transgenic crops wherein transgenics expressing an activator would be sprayed with the inactive precursor of an active (perhaps chemical) pesticide. Such a tactic could combine particular advantages of both the chemical and transgenic approaches to crop protection.

The only other reported successful example of pyramiding different insect control protein genes involved the toxins derived from Bt and scorpion venom which have been claimed to produce additive protective effects in transgenic plants (Barton and Miller, 1991).

3.3.2.8. Secondary metabolism. Many of the most potent protective compounds in plants are small, non-protein secondary metabolites (e.g. alkaloids, cyanogenic glycosides, glucosinolates, terpenoids, saponins etc.). These are usually the products of complex, multi-enzyme pathways. The effective manipulation of these metabolic pathways by the introduction (or elimination by anti-sense RNA technology) of enzyme encoding sequences, appropriately regulated, poses very considerable technical difficulties. Nevertheless, a number of laboratories are involved in attempting to modify existing pathways (e.g. Hallahan et al., 1992) although progress is understandably slow. The bioengineering of terpenoid metabolism in plants has been recently reviewed (McCaskill and Croteau, 1998) and well illustrates some of the problems with this approach. Terpenoids are produced from a single intermediate *via* complex, subtly regulated and compartmentalized pathways with many enzymes encoded by multigene families. Manipulation of the multiple pathway fluxes to specifically alter the final terpenoid profile without causing unintended deleterious effects on other end products is extremely complicated. The difficulties of successfully manipulating secondary metabolism are illustrated by the constitutive expression of phytoene synthase in transgenic tomato which has the expected result of increasing carotenoid accumulation but also results in a dwarf phenotype, explained by a depletion of the substrate which phytoene synthase shares with gibberellin synthesis (Fray et al., 1995).

A particular problem with this approach is that production of many of these secondary metabolites imposes a measurable 'cost' (yield penalty) on the host plants (see Vrieling et al., 1991). There is some evidence that such a 'cost' is not incurred by natural protection mechanisms based directly on protective proteins (Brown, 1988). It has been shown that expression of relatively large amounts of a non-phytotoxic foreign protein such as CpTI does not impose a yield penalty on transgenics (Hilder and Gatehouse, 1991) and most transgenics are described as being 'phenotypically normal'. Much of the

cost to plants of non-protein secondary compounds appears to be associated less with synthesis than with sequestration, detoxification, etc., of these compounds. Yield penalties could prove a further constraint on the development of this route to transgenic crop protection.

4. Outstanding problems with transgenic crop protection

A detailed coverage of the general problems of genetically manipulated organisms would be inappropriate in this article and we will concentrate on those issues of particular relevance to pest-resistant transgenics.

(i) *Public acceptability*: Recent surveys of farmers perception of insect resistant transgenic crops are generally favourable, at least in the US, with reduced exposure of farm-workers and the environment to insecticides perceived as major advantages of transgenics (Pilcher and Rice, 1998). However, there remains in many quarters serious (and growing) unease amongst the general public (i.e. the consumers) about transgenic crops in general and this encompasses crops which are genetically engineered for pest resistance. Some claim that this has led to excessive regulation as a result of 'self defensive responses of bureaucrats' (Miller, 1997). Public concerns are about the safety of genetically engineered foods and about the possible adverse impact of transgenic crops on the environment. An example of the former concern is that the antibiotic marker genes used to select for gene transfer might lead to antibiotic resistance in human pathogens. However, the generally accepted medical and scientific view is that the risk of compromising therapeutic antibiotics by the use of such markers is negligible (e.g. Flavell et al., 1992). Although methods have been developed for eliminating selectable marker genes after selection of transgenics (e.g. Yoder and Goldsbrough, 1994; Ebinuma et al., 1997), there seems little cause to encourage their widespread use. A further concern is the possibility of unexpected, harmful effects from introduced genes due to the random nature of the transformation process. Whilst there is a need to be aware of this possibility, the Food Industry does have a long and successful history producing safe food. However, the recent controversy in the UK over the genetically manipulated, lectin containing potatoes (Gillard et al., 1999) illustrates the need for new processing technologies (i.e. genetic engineering) to be rigorously tested for potential allergenic, toxic and antimetabolic effects in a transparent manner. Furthermore, entry into the public domain should be via the peer-reviewed process of the scientific press rather than through the media.

The main concern about the environment is the role of gene flow to weedy/wild relatives of crop plants. Transgenes can reach weed populations in viable pollen and, since most crops do have weedy/wild relatives somewhere, the escape of transgenes cannot be ruled out. Information on which to base realistic risk assessments is available for many regions (e.g. Raybould and Gray, 1993) and needs collecting for others to allow a case-by-case assessment to be carried out. The industry is aware of this and good agricultural procedures have been designed accordingly (see Boulter, 1995) in order to reduce the risks to an acceptable level.

There is a need for better, balanced presentation to and education of the general public concerning the risks/benefits of this technology and a greater public involvement in regulatory decision making (see Boulter, 1997). The public, supermarkets and farmers may have to agree to accept produce with some superficial insect damage when this has not led to reduced productivity. Again an understanding of the various trade-offs is required. Whilst organic food is not inferior in quality to non-organically grown food, it is produced by farmers who are willing to accept some insect presence in their fields.

Scientists also need to play, and be seen to play, their part in this newer, socially responsible scientific era. Technology 'chooses' simple, effective short term solutions, in this case synthetic chemical pesticides with complete insect kill, but which may in the longer term cause serious problems and there has been a reluctance to devise more subtle, long term sustainable IPM programmes. In practice, synthetic compounds with acute (toxic) effects will continue to play the prominent part in crop protection for the immediate future (see (ii) below).

(ii) *Efficacy*: Questions of the efficacy of transgenic crops depend very much on whether they are viewed from the perspective of chemical pesticides or from that of no additional protective intervention. Even the best current transgenics do not perform as spectacularly as chemicals. However, there have not yet been adequate trials of transgenics in genuine IPM systems. Such trials might be expected to demonstrate the real, long term benefits of transgenic crops, especially if factors such as environmental damage and health risks are included in the 'costings'. Such results might encourage greater enthusiasm for transgenics on the part of the users (i.e. farmers (see Palis,1998)).

The search for new, more-effective alternative genes is likely to focus on membrane bound targets (as with Bt toxins and lectins) rather than high-turnover digestive enzymes. Protein engineering may be brought to bear on increasing of effectiveness such membrane-binding agents, e.g. by attaching a cytolytic peptide such as an amphipathic α-helix (e.g. Jaynes et al., 1993) via an appropriate 'hinge' to a membrane-binding moiety.

(iii) *Spectrum of activity*: There are many insect pests which are simply not susceptible to the currently available range of ICP genes. Most prominent amongst these are the corn rootworms and cotton boll weevils, because of their high commercial value to agrochemical and seed companies in the US market (see Estruch et al., 1997).

Many very serious pests of local, crop-specific importance have received little or no attention from this technology, especially if no effective Bt has been found. Yet plant transformation technology is potentially very adaptable. There is a need to broaden the pool of (types of) genes which are available to cover these pests which are currently untreatable.

(iv) *Resistance management*: However effective a transgene might be initially, just as with chemical pesticides it is highly unlikely that the pests will not develop resistance to it. The durability of transgenics is likely to be extended with appropriate resistance management strategies. The broader the base of different types of genes which are available and the more readily alternatives can be developed, then the easier it will be to manage resistance effectively.

(v) *Commercialisation*: In a 1993 review (Boulter, 1993b) it was pointed out that there was a potential gap between academic expectations and commercial reality in this field. It was further suggested that it is likely that only the seed and agrochemical industries could carry out the necessary development work to show that the potential of ICPs such as those derived from plants was realisable commercially. More recently, Shewry and Lucas (1997) have highlighted this situation by lamenting the fact that 'no commercial cultivars have yet been produced expressing this novel source of resistance (CpTI), although the feasibility was established almost a decade ago'. Clearly their chronic action and inconsistency in expression have meant that the effects are not sufficient to lend confidence to commercialization.

Possible factors involved in commercialization considerations include:

(a) The costs to optimise and stabilise expression levels in field situations bearing in mind the relatively high levels of expression needed (of the order of 1% or more of soluble protein).

(b) The fact that the chronic rather than acute mode of action of many of these ICPs means they will best find their application in IPM programmes. Whilst this has long-term benefits relative to the use of synthetic chemicals, IPM is not popular with farmers (until its benefits have been demonstrated to them; see Palis, 1998) and has been somewhat neglected by scientists, even though this strategy might be the more profitable in the long term.

(c) Many of the companies which might develop this technology already produce synthetic chemical alternatives and have lobbied for regulations which would impose a serious market-entry barrier to genetically manipulated crops and garden plants. Miller (1999) has suggested that agricultural biotechnology is being seriously retarded by this over-regulation which may also have been responsible for some of the adverse public perception of transgenic products.

These considerations stress the need for education and a longer term, broader view and the need for governments and NGOs to pay for some of the research. It is perhaps ironic that EU regulations have reduced synthetic chemical pesticide usage whilst funding little research into the role of genetic engineering as part of an IPM programme.

5. The 'ideal' transgenic technology

Consideration of the current position of both the chemical pesticide and the transgenic crop approaches to crop protection has led us to analyse the characteristics which an 'ideal' new pesticide would possess. Amongst these we have identified:

(a) It should be relatively economical to produce.
(b) It should be environmentally benign (biodegradable).
(c) It should be easy to use in the field (especially if it is to be effectively and safely deployed in less sophisticated cropping regimes) and be deliverable specifically to the target site.
(d) It should have a wide spectrum of activity, but only against pests and not against associated beneficial insects or intended consumers.
(e) It should be generated by a technology which is flexible enough to allow any vulnerable site within the pest to be targeted; clearly target sites which have already become resistant to chemical pesticides are to be avoided.
(f) This technology should also be flexible enough to allow any particular pest species/order to be targeted.
(g) The technology should be adaptable enough to allow the ready development of alternatives if (when) resistance by the pest develops.
(h) It should preferably produce acute rather than chronic effects on the pest, although the value of the latter for use within an IPM strategy should be emphasised.

Transgenic crops would naturally meet the first few (a–c) of these criteria. We believe that the remaining criteria may be best met by exploiting genes which are based on antibody technology. Single-chain antibodies (ScFvs) would be used to block the function of essential pest proteins. The potential of plant-expressed antibodies or antibody fragments to serve as control agents against plant parasitic nematodes (Atkinson, 1993; Rosso et al., 1996), pathogenic viruses (Tavladoraki et al., 1993) or fungi (van Engelen et al., 1994) have been described. This antibody approach to the control of insect pests would offer the particular advantage of allowing some degree of selection of the specificity of effect, so that pests, but not beneficial insects could be specifically targeted. We anticipate that ScFvs would fit in well with the changed target site strategy set out in the following paragraph.

Meeting the final criterion, for acute effects, remains something of a problem. This may be seen as a shortcoming of the nature of the transgenic plant delivery system – presenting proteinaceous agents to the pests gut as part

of their food. As a target, the gut clearly presents especial difficulties for proteins. It is also well worth noting that the gut is not the target for any of the widely used synthetic chemical pesticides. These latter are usually targeted at more sensitive sites in the pest neurendocrine system, accessed from the haemolymph. The development of a delivery system from transgenic plants to pest haemolymph would remove a key constraint on the transgenic approach to crop protection. The need for this development is even more urgent in view of changes that have taken place in the pharmaceutical industry (see Margolis and Duyk, 1998) which is in many ways a weather-vane for the agrochemical industry.

The agrochemical industry is likely, albeit slowly, to follow the pharmaceutical industry in starting to change from chemistry-based whole organism screening, to biologically based target screening methods for product discovery. The use of biology, genomic tools and sequence information together with model insect and nematode systems such as *Drosophila* and *Caenorhabditis* opens up a new approach to pesticide discovery, replacing chemical modification and whole organism screening. This latter strategy has led over the years to the identification of few targets (75% of all chemical pesticides act on only two targets; see Margolis and Duyk, (1998)) and new approaches such as the biopesticides reviewed in this paper have their limitations.

Target based screening uses biological information to evaluate the suitability of target sites, which should have the following characteristics:

(a) viability,
(b) required throughout the life cycle,
(c) inhibition or hyper-activation should give knockdown,
(d) high sensitivity so that only partial interference would give knockdown,
(e) high genetic divergence to give protective selectivity,
(f) target easily formatted in high through-put assays.

The complete genomic sequences of model organisms will allow the eventual testing of all genes for suitability as targets using genetic screening, existing mutant collections and knockout screens.

6. Conclusion

Since the first reports of transgenic plants appeared in the early 1980s, there has been rapid progress directed at using this new technology for the very real, practical ends of crop protection. It is to be hoped that the encouraging progress described above is maintained and developed so as to make a significant contribution towards redressing the balance between world food productions and world food requirements in the coming century. Something has to! The technology developed will have to take a broader, longer term, more socially responsible approach than is usually the case now and scientists, industrial and government administrators, farmers and the general public all have roles to play.

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