Recent developments and future prospects in insect pest control in transgenic crops

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Abstract
Adoption of insect-resistant transgenic crops has been increasing annually at double digit rates since the commercial release of first generation maize and cotton expressing a single modified Bacillus thuringiensis toxin (Bt) nine years ago. Events have shown that these crops can be successfully deployed in agriculture, where they decrease pesticide usage, and are environmentally benign. Sustainability and durability of pest resistance remain a constant point for discussion. This review focuses on the science that underpins second, and third generation insect-resistant transgenic plants. It also examines the appropriateness and relevance of models that are currently being used to determine deployment strategies, to maximize sustainability and durability. Finally, it reviews recent strategies that are being developed for novel approaches for transgenic insect pest control.

Transgenic crops carrying single insect resistance genes: a brief survey of recent economic and deployment data

Many excellent accounts of the economic, environmental and health benefits of insect resistant transgenic crops have been published [1,2] In The US the six biotechnology-derived crops planted in 2003 (canola, corn, cotton, papaya, squash and soybeans) produced an additional 5.3 billion pounds of food and fibre and increased farm income by $1.9 billion. These biotechnology-derived crops also reduced the use of pesticides by 46.4 million pounds [3]. The current status of Bt rice, which is expected to be commercially released in China in 2006, is reviewed by High et al. [4]. A number of GM (Genetically Modified) rice varieties have entered and passed field and environmental release trials, and 4 varieties entered preproduction trials in farmers’ fields in 2001. Farm surveys of randomly selected households cultivating insect-resistant GM rice varieties demonstrate that when compared with households cultivating non-GM rice, small and poor farm households benefit from adopting GM rice by both higher crop yields and reduced use of pesticides, which also contribute to improved health. For rice, the development and implementation of appropriate resistance management strategies, and resolution of trade policy barriers, are key constraints that have delayed earlier widespread cultivation of the crop [5]. For cotton, key documented benefits are a 70% reduction in insecticide applications in Bt cotton fields in India, resulting in a saving of
up to US$30 per hectare in insecticide costs, with an increase of 80-87% in yield of harvested cotton [6] and a dramatic reduction in pesticide applications in Bt cotton fields in China. The same survey revealed that the percentage of farmers with pesticide poisoning was reduced from 22% to 4.7% [7].

Field evaluation to assess potential hazards of growing Compa®, a transgenic Bt maize variety based on the transformation event CG 00256-176 was performed in Spain [8]. Two categories of potential hazards were investigated: the potential of the target corn borer *Sesamia nonagrioides* to develop resistance to Bt maize and effects on non-target species (herbivores and predators). Larvae collected in Bt fields at later growth stages, in which event 176 Bt maize expresses the toxin at sublethal concentrations, had longer diapause and post diapause development than larvae collected in non-Bt fields, a feature that might lead to a certain isolation between populations in both type of fields and accelerate Bt resistance development. Transgenic maize did not have a negative impact on non-target pests in the field or on natural predators; more aphids and leafhoppers but similar numbers of cutworms and wireworms were counted in Bt versus non-Bt fields.

Development of “second generation” resistant crops continues; for example, transgenic maize plants resistant to corn rootworm were first commercialized in the US in 2003. The commercialized event, MON863 was developed using a synthetic variant of the wild type Cry3Bb1 gene from *Bacillus thuringiensis kumamotoensis* that encodes a protein with eight times enhanced insecticidal activity [9]. The gene was codon-optimized for optimal expression in monocotyledonous plants, was expressed under the control of the root-enhanced 4AS1 promoter and introduced into corn cell cultures using particle bombardment. Maize hybrids containing MON863 are more efficacious than soil and seed applied insecticides in protecting roots of corn plants from larval feeding damage.

**Predictive models for deployment and resistance management: appropriateness and relevance**

Some commentators predicted that Bt-insect resistant crops would be of limited durability, due to selection of mutations present at low frequency in “wild” pest populations which give tolerance to the toxins. However, no such tolerance to the toxin (normally referred to as “resistance”) has been observed during the 9 years of commercial deployment. An 8 year monitoring study of pink bollworm resistance to Bt toxin with laboratory bioassays of strains derived annually from 10-17 cotton fields in Arizona showed no net increase from 1997 to 2004 in the mean frequency of bollworm resistance to Bt toxin [10]. Similarly, a large scale survey carried out in Bt maize fields in Spain did not detect any resistant corn borers (*Sesamia nonagrioides* and *Ostrinia nubilalis*) over a 5 year period [11]. Interestingly, it was possible to select resistant populations of the two borers in the laboratory. This reflects the fact that laboratory data should be treated with caution in terms of extrapolating these to a field setting. It is not uncommon to observe resistant insects under laboratory conditions. This is discussed in detail in subsequent sections.

Different hypotheses have been propounded to support the predictions for the nature and timing of resistance development in field populations of target insects (Box 2). One such hypothesis which gained more credibility is that Bt resistant insects would quickly develop in transgenic cotton and maize unless massive refuges were instituted. The
refuge deployment strategy has thus been widely adopted, but has been criticized [12]. Most hypotheses assumed a single binding site for the toxin and minor unfitness of resistant individuals; and did not recognize that resistant individuals might be extremely unfit, or that Bt might have multiple targets [13-15]. Under conditions where insects have to tolerate high levels of Bt, it is likely that the unfitness of resistant mutations is so high that resistance is effectively lethal in the field if the selection pressure is removed, especially if the compounded unfitness of mutations at more than one gene is considered [12]. Obviously, hypotheses should be subject to criticism, and not irreversibly accepted as being axiomatic. A highly pertinent example of such (self)-criticism is a recent report by Gahan et al., who have modified their stand on insect resistance to Bt from claiming it to being mediated by a single gene [16] to suggesting it as a quantitative trait [17].

The failure to observe resistance to Bt-expressing transgenic crops in the field needs to be reconciled with the relative ease of developing resistant insect populations in the laboratory, and the development of field resistance to Bt-sprays in insect populations. Resistance to Bt toxins supplied in artificial diets or in leaf dip bioassays does not necessarily result in the development of insect populations that can survive on transgenic plants expressing Bt toxins [18]. For example, a highly resistant strain of the European corn borer (Ostrinia nubilalis) that was selected with 70-fold resistance to a formulation of 4 different Bt toxins was unable to survive on transgenic corn expressing some of these proteins [19]. Similarly, Cry3A-resistant Colorado potato beetle (induced by feeding the toxin to neonates in artificial diets) was not able to survive on Bt potato plants expressing the same toxin [20] and Cry1Ac-resistant corn earworm (Heliothis virescens) did not survive on Bt cotton. However, a number of highly resistant strains of the diamondback moth and the pink bollworm were able to survive on Bt-transgenic crops, in the laboratory. Field evaluation of resistance is necessary since environmental conditions that affect fitness cannot be mimicked in the laboratory; indirect experiments indeed demonstrated such fitness differences [18, 21].

The accepted method for preventing the development of resistance to Bt crops in insect pests is the refuge strategy, in which a proportion of the total area used for growing the crop is given over to plants which are susceptible to the pest (wild type). The refuge plants serve to maintain a population of the pest which does not carry any resistance mutation and can “dilute out” a resistance allele; the chances of individuals whose genotype is homozygous for resistance arising is thus kept low. The refuge strategy has proved successful in that pest resistance to Bt has yet to become a problem. However, there is an adequate body of evidence to argue scientifically against the need or usefulness of refuges in the context of resistance management (see references in [12]). This view, although not “politically correct”, does suggest that failure to adhere strictly to the refuge method for resistance management, which is likely to occur with less controlled and more widespread growing of transgenic crops, will not necessarily lead to rapid breakdown in the usefulness of Bt crops.

Theoretical models predict that plants expressing two dissimilar Bt toxin genes are likely to have the potential to delay resistance in target insect populations more effectively than single toxin-containing plants [22]. A model system consisting of Bt transgenic broccoli plants expressing Cr1Ac and Cry1C and the diamondback moth, Plutella xylostella was used in greenhouse studies incorporating a 20% refuge plants [23]. The moth carried genes for resistance to both, one or neither of the toxins. The objective of the study was
to determine how rapidly such an insect population that contains a relatively high frequency of alleles for resistance to Cry1Ac and Cry1C is able to develop resistance to each or both toxins when exposed to plants that express both toxins simultaneously. After 24 generations of selection, resistance to pyramided two-gene plants was significantly delayed when compared with resistance to single-gene plants and to Cry1Ac toxin. Few Cry1Ac-resistant pink bollworms survived on plants expressing both Cry1Ac and Cry2Ab [24]. However, it was found that the two bolls from which the survivors emerged in a plant that contained Cry1Ac and Cry2Ab did not express Cry2Ab. As the bollworm strain used was already selected on Cry1Ac, the survivors did not actually indicate resistance to Cry2Ab.

It should be noted that dual Bt gene plants require less refuge and now companies are trying to eliminate structured refuges when dual Bt gene plants are being used. Monsanto recently petitioned US EPA to eliminate the non-Bt structured refuge requirement for farmers in Texas, the Mid-South and the Southeast when they grow Bollgard II Bt cotton, and use natural refuge plants for resistance management [25].

**Effects of insect-resistant transgenic crops on non-target beneficial insects in the context of IPM**

A major tactic of IPM (Integrated Pest Management) is to preserve natural enemies associated with crop pests [26]. Tritrophic interactions involving crops, insect pests and their natural enemies must be taken into account when evaluating the environmental impact of transgenic crops. Natural enemies of pest species include generalist predators such as carabid beetles [27] or specific parasites such as parasitoid wasps [28]. Although insect-resistance factors expressed in crops might not have a direct effect on natural enemies of pests, indirect effects are almost inevitable. For example, prey fed on plant material expressing Bt proteins were compromised in development and consequently nutritional quality, and affected growth and development of a carabid beetle, with early instars being more sensitive than later instars and adult beetles [29]. The Bt toxin did not accumulate through the different trophic levels in these experiments, but was excreted. Studies such as these can be criticized on the grounds that they over-estimate negative effects on non-target insect populations, and over-emphasize their importance in comparison to other environmental factors, which are known to have much more substantial effects on the performance of predators and parasites than the effects observed as a result of feeding Bt toxin to prey in the laboratory. Biological relevance, rather than mathematical significance, has to be determined to evaluate ecological impacts [30].

The effects of insect-resistant crops on non-target insects should also be evaluated in the right context, i.e. in comparisons with other measures farmers take to control insects [31]. The use of traditional chemical pesticides affects benign insects directly, and in comparison the specificity and absence of direct effects on biological control agents and non-targets make transgenic crops expressing Bt more environmentally friendly. All measures to protect crops against insect pests will reduce the numbers of available prey for predators and parasites, even if there is no direct effect [32].
The new frontier: second and third generation insect resistant plants

Constitutive or tissue specific expression?
Whilst constitutive expression of insecticidal transgene products has provided high levels of resistance in crop plants, tissue-specific or inducible expression might be desirable under some circumstances. Since the epidermal cells are the first to be attacked by insects, defence genes expressed under epidermal cell specific promoters, e.g. CER6 an enzyme for cuticular wax production [33], might be useful. Phloem feeding insects can be targeted using the root phloem-specific promoter AAP3 [34], the phloem-specific pumpkin promoter PP2 [35] and the rice sucrose synthase Rss promoter [36]. Currently progress is being made with chemically inducible promoters including those induced by ethanol [37], tetracycline, copper, glucocorticoid steroid hormones and steroidal, and non-steroidal, ecdysone agonists [38,39]. Creating ‘within-plant refuge’ is a novel application of using inducible promoters whereby the transgenic plant or parts thereof can serve as a refuge plant as long as either the expression of the insecticidal gene is not induced or the induction wears off [40]. The role of transcription factors (TFs) in controlling gene expression has not been fully exploited. A single TF can affect multiple pathways and potentially activate multiple endogenous resistance mechanisms. A novel use of pest TFs is exemplified by transgenic plants expressing a molting related TF under tissue-specific and/or inducible promoters [41]. The TF EcR that serves as an ecdysone receptor binds to the steroid hormone and DNA through a EcR-USP (ultra spiracle) heterodimer, initiating the expression of genes involved in the molting process. Transgenic plants expressing these TFs cause insect larvae feeding on them to undergo faulty and/or lethal molting.

Transgenic plants with multiple resistance genes
The simultaneous introduction of three genes expressing insecticidal proteins, Cry1Ac, Cry2A and Gna into indica rice to control three major pests, rice leaf folder (Cnaphalocrocis medinalis), yellow stem borer (Scirpophaga incertulas) and the brown planthopper (Nilaparvata lugens) has been reported [42]. The leaf folder and the stem borer are targeted by the Bt genes while the planthopper is targeted by Gna. Triple transgenic plants were more resistant compared to their binary counterparts. Comparison of three different transgenic Bt cotton populations containing either the single Cry1Ac or Cry2Ab, or both genes, for fruit penetration and damage by a feral and a Cry1Ac-selected strain of cotton bollworm revealed that transgenic cotton containing two Bt genes performed better [43]. These are few examples where transgene pyramiding was used in a crop plant to create durable resistance against multiple insect pests with different feeding modes.

Domain swapping in cry toxins
Most activated Cry toxins share a common three-domain structure [44]. The N-terminal domain I, is believed to insert into the target membrane and form part of a membrane pore; domain II is implicated in receptor binding and thus determines in part specificity to particular insects, and the C-terminal domain III is also involved in specificity through receptor binding. Various investigators demonstrated that hybrid Cry toxins exhibited substantially enhanced toxicity or host range. Enhanced efficacy of Bt Cry proteins was
achieved by creating fusions between domain III of Cry1Ac and domains I and II of various other Cry1 proteins [45]. Similarly, a hybrid toxin was developed against Spodoptera litura, a polyphagous pest that is tolerant to most Bt toxins [46]. A poorly active domain in the naturally occurring Cry1Ea toxin was replaced with a highly homologous 70 amino acid region of Cry1Ca in domain III. The synthetic gene was further optimized for high level expression in plants and was introduced into tobacco and cotton plants. Resulting plants were found to be extremely toxic to Spodoptera litura at all stages of larval development.

A hybrid Bacillus thuringiensis gene was constructed using a synthetic and truncated cry1Ba gene as the scaffold for inserting part of cry11a encoding domain II [47]. Transgenic potato plants expressing this hybrid toxin were resistant to a number of insect pests including both Coleoptera (Colorado potato beetle) and Lepidoptera (potato tuber moth and European corn borer). As the target receptor recognition of this hybrid protein is expected to be different from Cry proteins currently in use to control these pests, this strategy provides new opportunities for resistance management studies involving multiple transgenes in crops.

Plant derived lectins and their roles in insect pest control

The ability of the mannose-specific snowdrop lectin (Galanthus nivalis agglutinin: GNA) to serve as a carrier protein to deliver insecticidal peptides and proteins to the haemolymph of lepidopteran larvae was demonstrated by expressing GNA-allatostatin and GNA-SFI1 fusions in Pichia pastoris and using the purified fusion protein in artificial diets against the tomato moth, Lacanobia oleracea [48, 49]. SFI1 is an insecticidal venom neurotoxin from the spider Segestria florentina. Whereas the two individual components of the toxins showed no oral toxicity, the fusion proteins were insecticidal to lepidopteran larvae.

Unmodified lectins have been shown to be insecticidal towards sap-sucking insects outside the host range of Bt; for example, garlic (Allium sativum) leaf lectin expressed in transgenic tobacco plants substantially decreased survival of the peach potato aphid (Myzus persicae) [50].

Engineering plants with a fusion protein combining Cry1Ac with the galactose-binding domain of the non-toxic ricin B-chain provides the toxin with additional, binding domains, thus increasing the potential number of interactions at the molecular level in target insects. Transgenic rice and maize plants engineered to express the fusion protein were significantly more toxic in insect bioassays than those containing the Bt gene alone [51]. They were also resistant to a wider range of insects, including important pests that are not normally susceptible to Bt toxins. The recognition of toxin binding sites in the insect midgut is an important factor determining the spectrum of Bt toxin activity and severity of toxemia (references in [51]).

New strategies employing protease inhibitors

Transgenic plants expressing protease inhibitors have so far shown marginal effectiveness against insect pests. Reasons for this lack of effectiveness include the adaptive capacity of gut proteolysis in phytophagous insects, based on genetic diversity in proteinases, and low potency of specific protease inhibitors that exhibit insecticidal activity. Even combined use of two such inhibitors, the potato PI-II and the
carboxypeptidase (PCI) inhibitors was not adequate to prevent this compensatory response [52]. Nevertheless, PIs have the potential to be effective insecticidal proteins if insect adaptation to them can be overcome; the use of novel inhibitors, such as the barley trypsin inhibitor (BTICMe [53]) equistatin from sea anemone [54], other cystatins [55, 56] or synthetic constructs containing multiple inhibitors [57] or inhibitors and lectins [58] might prove useful.

**Non conventional sources for insect resistance**

Second generation insect-resistant transgenic plants with increased potential for durable resistance might result from the deployment of plants expressing multiple insecticidal novel proteins such as the Vip or vegetative insecticidal proteins produced by *Bacillus thuringiensis* during its vegetative growth. These have insecticidal activity towards a wider spectrum of insect pests, yet they have little sequence homology with the more conventional Cry proteins [59, 60]. Transgenic cotton expressing such a Vip protein is expected to be released commercially in the US during 2006. *Photorhabdus* and *Xenorhabdus* bacteria are symbionts of entomopathogenic nematodes. Unlike Bt toxins, proteins produced by these two bacteria are not acutely toxic when ingested by the insect, rather they cause septicemia in the insect, the insect is killed and its tissues are used as nutrients by the nematode [61]. Considerable progress has been made in the identification of several toxin genes from these two bacteria [62]. These genes encode large insecticidal toxin complexes with little homology to other known toxins. Arabidopsis plants expressing the toxin A gene from *Photorhabdus luminescens* had good insecticidal activity against one lepidopteran and moderate activity against a coleopteran pest [63].

**Contribution of endogenous resistance mechanisms to crop protection against insect pests – non host resistance and signalling**

All plants have some level of endogenous resistance to attack by insect pests. However, as a result of co-evolution, herbivorous insects have adapted to plant defences by evasion and/or detoxification (general review in [64]). Insects are also able to compromise defence strategies by exploitation of signalling mechanisms. Corn earworm uses signalling molecules such as jasmonate and salicylate, from its plant host, to activate four of its cytochrome P450 genes, making the induction of detoxifying enzymes rapid and specific [65]. These shifts in plant-pest responses highlight the complexity of the interaction, in which the insects’ ability to overcome induced resistance has a role in determining whether it is able to be a successful herbivore [66].

The existence of shared plant defence response strategies/pathways against pathogens and pests suggests that knowledge of non-host resistance mechanisms against pathogens in crops could be exploited to improve pest resistance. For example, Biere *et al.*, show that plants selected for high levels of iridoid glycosides showed resistance *in vivo* to both a generalist herbivore (*Spodoptera*) and a fungal pathogen (*Diaporthe adunca*) [67]. An anti-fungal triterpenoid saponin in the crucifer *Barbarea vulgaris* gives resistance to the lepidopteran *Plutella xylostella* (a crucifer specialist, which is not affected by the main defensive compounds in these plants, the glucosinolates) [68]. Non-host resistance is thought to be multigenic and the inactivation of any one component might not be sufficient to render a plant susceptible [69]. Up-regulation of defence and non-host
resistance mechanisms of the host plant can make plants tolerant or resistant to multiple pests. Naeemullah et al. showed in rice that plant resistance to one insect (brown plant hopper; *Nilaparvata lugens*) might deter another (*Plodia interpunctella*) [70]. Processes like redox signalling are similar in biotic and abiotic stresses, and there is an underlying cross-talk between the responses resulting from pest attack, pathogen attack and various abiotic factors such as drought, salinity and mechanical damage. For example, genes such as inositol-phosphatase and ADP-ribosylase, known to have roles in calcium and abscisic acid mediated signalling in abiotic stress [71] are also upregulated in response to pests [72]. A better understanding of molecular events in responses to biotic stresses will allow manipulation of genes, gene expression patterns or growth conditions that facilitate resistance to one or more pests through endogenous mechanisms.

**Conclusions**
Experience has shown the benefits of transgenic insect-resistant crops in terms of increased yields, reduced chemical inputs and, as a knock-on effect, improved farmer and consumer health. On the other hand, although there has been no evidence for detrimental effects, the potential for development of pest resistance, and indirect damage to non-target species, call for reason and caution in how we deploy transgenic plants expressing insecticidal genes. This, however, does not translate to a *de facto* moratorium on research to improve the “first generation” insect resistant transgenic crops. On the contrary, bold and daring strategies need to be explored to test hypotheses and arrive at strategies that provide an overall balance of cost versus benefit. The future security of food supply will depend on science providing the tools to allow efficient agricultural production, which is sustainable in every sense, to continue; transgenic insect-resistant plants have a track record of success which will become progressively more difficult for opponents of genetic engineering technology to ignore.

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**Box 1. Glossary:**
First generation transgenic plants: Transgenic plants containing only marker genes useful in the development of transformation systems.
Second generation transgenic plants: Transgenic plants containing, in addition to the selectable marker one or two transgenes encoding simple agronomic traits (such as pest and herbicide resistance).
Third generation transgenic plants. Transgenic plants which contain multiple transgenes targeting multiple pests and diseases, often in a temporal/spatial manner. These might also express additional value-added or agronomic traits.
Box 2. Proposed reasons leading to the development of insect populations resistant towards Bt proteins:

1. Naturally mutated host genes [73]
2. Synthetically mutated genes [74]
3. Loss of midgut proteases required to activate protoxins [75]
4. Higher gut proteolytic activity leading to toxin degradation
5. Reduction in binding affinity dependant on membrane integrity [15]
6. Cell-cycle-dependent absence of membrane lipid raft domains that are essential for binding the toxin [76]

References


15. Avisar, D. et al. (2004) The role of *Bacillus thuringiensis* Cry1C and Cry1E separate structural domains in the interaction with *Spodoptera littoralis* gut epithelial cells. *J. Biol. Chem.* 279, 15779-15786


17. Gahan et al., (2205) Genetic basis of resistance to Cry1Ac and Cry2Aa in *Heliothis virescens* (*Lepidoptera : Noctuidae*) *J. Econ. Entom.* 98: 1357-1368


28. Vojteck, E. *et al.* (2005) Effects of Bt maize on the herbivore *Spodoptera littoralis* (Lepidoptera: Noctuidae) and the parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae) *Transgen Res* 14, 133-144


44. de Maagd, R.A. et al. (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Genet.* 17, 193-199

45. Karlova, R. et al. (2005) *Bacillus thuringiensis* delta endotoxin Cry1Ac domain III enhances activity against *Heliothis virescens* in some, but not all Cry1-Cry1Ac hybrids. *J Invert Pathol.* 88, 169-172


69. Yun, B.W. et al. (2003) Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in Arabidopsis against wheat powdery mildew Plant J 34, 768-777


72. Qubbaj, T. et al. (2005) Molecular interactions between rosy apple aphids, Dysaphis plantaginea, and resistant and susceptible cultivars of its primary host Malus domestica Entomol Exp et Appl 115, 145-152


