

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

Paul Christou¹, Teresa Capell¹, Ajay Kohli², John A. Gatehouse³ and Angharad M.R. Gatehouse²

¹ICREA, Universitat de Lleida, PVCF, Av Alcalde Rovira Roure, 191, E-25198, Lleida, Spain

²IRES, Devonshire Building, University of Newcastle, Newcastle upon Tyne, NE1 7RU, UK

³School of Biological and Biomedical Sciences, Durham University, South Road, Durham DH1 3LE, UK

The 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. Studies have shown that these *Bt* crops can be successfully deployed in agriculture, which has led to a decrease in pesticide usage, and that they are environmentally benign. However, the sustainability and durability of pest resistance continues to be discussed. In this review, we focus on the science that underpins second- and third-generation insect-resistant transgenic plants and examine the appropriateness and relevance of models that are currently being used to determine deployment strategies to maximize sustainability and durability. We also review strategies that are being developed for novel approaches to 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 USA, the six biotechnology-derived crops planted in 2003 (canola, corn, cotton, papaya, squash and soybean) produced an additional 2.4 million tonnes of food and fibre and increased farm income by US\$1.9 billion. These biotechnology-derived crops also reduced the use of pesticides by 21 000 tonnes [3]. The current status of *Bt* rice expressing the *Bacillus thuringiensis* toxin, which is expected to be commercially released in China in 2006, is reviewed in Ref. [4]. Several genetically modified (GM) rice varieties have entered and passed field and environmental release trials, and four varieties entered preproduction trials in farmers' fields in 2001. Farm surveys of randomly selected households cultivating insect-resistant GM rice varieties indicate that GM rice cultivation is

beneficial to small and poor farm households because crop yields are higher and pesticide use is reduced, which has also contributed to improved health, compared with households cultivating non-GM rice. For rice, the development and implementation of appropriate resistance management strategies, and resolution of trade policy barriers, are key constraints that 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 ha in insecticide costs, and an 80–87% increase in harvested cotton yield [6]; a dramatic reduction in pesticide applications in *Bt* cotton fields has also been reported in China, and the proportion of farmers with pesticide poisoning has been 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 the effects on non-target species (herbivores and predators). Larvae collected from fields in which event 176 *Bt* maize (which expresses the toxin at sublethal concentrations) was grown had longer diapause and post-diapause development than larvae collected in fields containing non-*Bt* maize. This feature might lead to pest populations in fields containing transgenic and non-transgenic plants to become isolated from each other, and might accelerate development of resistance to *Bt*. Transgenic maize did not have a negative impact on non-target pests in the field or on natural predators: more

Glossary

First generation transgenic plants: transgenic plants containing only marker genes, which are 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 that contain multiple transgenes targeting multiple pests and diseases, often in a temporal or spatial manner. These might also express additional value-added or agronomic traits.

Corresponding authors: Christou, P. (paul.christou@icrea.es), Gatehouse, J.A. (j.a.gatehouse@durham.ac.uk).

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aphids and leafhoppers were counted in *Bt* versus non-*Bt* fields but similar numbers of cutworms and wireworms were present in both fields.

The development of 'second generation' resistant crops continues (see Glossary): for example, transgenic maize plants resistant to corn rootworm were first commercialized in the USA 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 that has eight times more insecticidal activity than the wild type [9]. The gene was codon-optimized for optimal expression in monocot 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 have predicted that *Bt*-insect-resistant crops would be of limited durability because mutations present at low frequency in 'wild' pest populations would be selected and 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 (1997–2004) of pink bollworm resistance to *Bt* toxin with laboratory bioassays of strains derived annually from 10–17 cotton fields in Arizona (USA) showed no net increase 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 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 proposed to support the predictions for the nature and timing of resistance development in field populations of target insects (Box 1). One such hypothesis that has gained more credibility than others 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 but 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, particularly 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 Linda Gahan *et al.*, who have modified their stand on insect resistance to *Bt* from claiming that it is mediated by a single gene [16] to suggesting that it is a quantitative trait [17].

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*), selected on a formulation of four different *Bt* toxins, showed a 70-fold increase in the levels of toxin required for mortality compared with wild type, but 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, several 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 because 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 that are susceptible to the pest (wild type). The refuge plants serve to maintain a population of the pest that 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 sufficient evidence to argue scientifically against the need or usefulness of refuges in the context of resistance management (Ref. [12] and references therein). 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

Box 1. Hypotheses for insect populations developing resistance towards *Bt* proteins

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

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 where 20% of the plants were 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 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.

Because dual *Bt* gene plants require less refuge, companies are trying to eliminate structured refuges when dual *Bt* gene plants are being used. Monsanto recently petitioned the US Environmental Protection Agency 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 Integrated Pest Management

A major tactic of Integrated Pest Management (IPM) 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, which affected the 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 that 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. compared with other measures farmers take to control insects) [31]. Compared with traditional chemical pesticides, which affect benign insects directly, transgenic crops expressing *Bt* are more environmentally friendly because of their specificity and absence of direct effects on biological control agents and non-targets. 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?

Although 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. Because 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]. 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 a '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, which serves as an ecdysone receptor, binds to the steroid hormone and DNA through an 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 *Bt* genes target the leaf folder and the stem borer, and the *Gna* gene targets the planthopper. Triple transgenic plants were

more resistant compared with their binary counterparts. Comparison of three different transgenic *Bt* cotton populations containing either the single Cry1Ac or Cry2Ab gene, 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 a 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, in part, determines specificity to particular insects. 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 cryIIa gene encoding domain II [47]. Transgenic potato plants expressing this hybrid toxin were resistant to several 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 the percentage survival of peach potato aphids (*Myzus persicae*) feeding on these plants compared with controls [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 attack by 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 (Ref. [51] and references therein).

New strategies employing protease inhibitors

Transgenic plants expressing protease inhibitors have to date 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 (PI) 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 also 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 (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 is expected to be released commercially in the USA 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. Instead they cause septicaemia 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 toxin A gene from *Photorhabdus luminescens*

showed strong 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 [64]. Insects are also able to compromise defence strategies by exploiting signalling mechanisms. The 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 ability of the insect 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 or 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, 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 glucosinolates, the main defensive compounds in these plants) [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. For example, in rice, resistance to one insect (brown plant hopper, *Nilaparvata lugens*) can deter another (*Plodia interpunctella*) [70]. Processes such as 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, which are 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 that are triggered in response to biotic stresses should enable the 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. However, although there has been no evidence for detrimental effects, the potential

for pest resistance to develop and indirect damage to non-target species call for reason and caution in how we deploy transgenic plants expressing insecticidal genes. This does not translate to a 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 to continue that is sustainable in every sense: transgenic insect-resistant plants have a track record of success that will become progressively more difficult for opponents of genetic engineering technology to ignore.

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