Biotechnological Prospects for Engineering Insect-Resistant Plants[1][C]

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Insect-resistant crops have been one of the major successes of applying plant genetic engineering technology to agriculture; cotton (*Gossypium hirsutum*) resistant to lepidopteran larvae (caterpillars) and maize (*Zea mays*) resistant to both lepidopteran and coleopteran larvae (rootworms) have become widely used in global agriculture and have led to reductions in pesticide usage and lower production costs (Toenniessen et al., 2003; Brookes and Barfoot, 2005).

The source of the insecticidal toxins produced in commercial transgenic plants is the soil bacterium *Bacillus thuringiensis* (*Bt*). *Bt* strains show differing specificities of insecticidal activity toward pests, and constitute a large reservoir of genes encoding insecticidal proteins, which are accumulated in the crystalline inclusion bodies produced by the bacterium on sporulation (Cry proteins, Cyt proteins) or expressed during bacterial growth (Vip proteins). The three-domain Cry proteins have been extensively studied; their mechanism of action involves a proteolytic activation step, which occurs in the insect gut after ingestion, followed by interaction of one or both of domains II and III with ''receptors'' on the surface of cells of the insect gut epithelium. This interaction leads to oligomerization of the protein, and domain I is then responsible for the formation of an open channel through the cell membrane (Bravo et al., 2007). The resulting ionic leakage destroys the cell, leading to breakdown of the gut, bacterial proliferation, and insect death.

However, not all pests are adequately targeted by the *Bt* toxins used at present, and there is still a need to develop solutions to specific problems, such as resistance to sap-sucking pests and pests of stored products. This *Update* will review some developments to the basic *Bt* strategy and selected alternative methods for engineering insect resistance.

MORE OF THE SAME? IMPROVING EXPRESSION LEVELS OF *Bt* TOXINS, "GENE STACKING" (MULTIPLE TOXINS), AND NEW *Bt* TOXINS

Plastid Genome Transformation

Expression of *Bt* toxins in transgenic plants needs to be at a sufficient level to confer adequate protection against target pests (defined by the Environmental Protection Agency as giving >95% mortality of insects heterozygous for a resistance allele; in practice, generally >0.2% of total soluble protein in the appropriate tissue). Transformation of the nuclear genome with genes encoding *Bt* toxins gives very low levels of expression unless extensive modifications, which include removal of AT-rich regions from the coding sequence and use of modified constitutive or tissue-specific promoters, are carried out. These methods were established within the first stage of the development of this technology and are now considered routine, although they do pose significant technical problems.

In contrast, introduction of unmodified *Bt* genes into the chloroplast genome results in high levels of toxin accumulation (3%–5% of total leaf protein; McBride et al., 1995), as the plastid genome is bacterial in origin. This method has not been widely adopted, due to significant technical problems in achieving stable transformation of the plastid genome and in transforming plastids in species other than tobacco (*Nicotiana tabacum*). Nevertheless, these difficulties can be overcome; various Cry proteins have been expressed in plastids of tobacco (Kota et al., 1999; De Cosa et al., 2001), and Cry1Ab has been expressed in soybean (*Glycine max*) plastids (Dufourmantel et al., 2005). Overexpression of the Cry2Aa2 operon in plastids is effective in giving broad-spectrum protection against a range of pests. This technique has the potential advantage that plastid-encoded characteristics are predominantly maternally inherited in most plants, so that pollen from transgenic crops is less likely to disperse the transgene to nontransgenic plant stocks. The inherent containment addresses concerns about the coexistence of transgenic and organic agricultural practices and may lead to this technique being more widely adopted for production of agricultural crops.

Novel *Bt* Toxins

Several novel *Bt* insecticidal proteins, which have no sequence similarity to three-domain Cry proteins,

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have been expressed in transgenic plants. Binary toxins require two components for activity and are exemplified by the Cry34/35 and Vip1/2 toxins, which are active against corn rootworm (*Diabrotica virgifera*). Cry34/35 have been expressed in transgenic maize (Moellenbeck et al., 2001) and are the basis of a successful commercial product; no re-engineering of coding sequences was necessary, in contrast to an alternative approach using modified Cry3Bb1 (see below). Single-chain Vip proteins, such as Vip3, are active against lepidopteran larvae, with a broader range of toxicity than individual Cry proteins. Their range of activity can be further extended by protein engineering (Fang et al., 2007). Commercial transgenic plants expressing these proteins are under development (Christou et al., 2006).

Plants Expressing Multiple Toxins

The specificity of *Bt* Cry toxins toward target pest species is a major advantage in agriculture because effects on nontarget insects and other organisms in the ecosystem are minimized. However, deployment of transgenic crops expressing a single specific *Bt* toxin can lead to problems in the field, where secondary pest species are not affected, and can cause significant damage to the crop. Introduction of additional *Bt cry* genes into the crop can afford protection against a wider range of pests. Commercial use of transgenic cotton containing two *Bt* genes began in 1999, 3 years after the release of the original single *Bt* variety. Cotton plants expressing both Cry1Ac and Cry2Ab proteins were more toxic to bollworms (*Helicoverpa zea*; target pest) and two species of armyworms (*Spodoptera frugiperda* and *Spodoptera exigua*; secondary pests) than cotton expressing Cry1Ac alone in laboratory trials (Stewart et al., 2001) and in greenhouse and field trials (Chitkowski et al., 2003).

Expression of multiple Cry proteins can also be beneficial in prevention of resistance to toxin activity in the target pest(s). Although the ''approved'' refuge strategy has been highly successful in containing pest resistance to *Bt* toxins expressed in transgenic plants (Tabashnik et al., 2005), targeting different receptors in the insect is (theoretically) more effective because multiple mutations are required to produce the loss of sensitivity to the toxins. Transgenic broccoli plants expressing either Cry1Ac or Cry1C or both proteins were exposed to a population of diamondback moth (*Plutella xylostella*), which carried *Bt* resistance genes at a relatively low frequency (Zhao et al., 2003). After 24 pest generations, plants carrying two transgenes showed a significant delay in the selection of a resistant population of insects. However, simultaneous exposure of the insects to plants carrying either one or two *Bt* genes actually increased resistance breakdown in the two gene plants (Zhao et al., 2005), suggesting that the multiple-toxin approach is not a cure-all solution. Other results have also shown that pests can acquire resistance to multiple toxins; for example, a strain of the lepidopteran cotton pest *Heliothis virescens* has simultaneous resistance to Cry1Ac and Cry2Aa, with a different genetic basis of resistance to each toxin (Gahan et al., 2005).

Improvements in plant transformation methods, such as extending the species range of *Agrobacterium*-mediated gene transfer methods to monocots and using plasmid vectors containing multiple gene constructs to allow introduction of multiple transgenes at a single genetic locus, have enabled the expression of multiple toxins in transgenic plant varieties. The recent announcement of a transgenic maize variety containing six insect resistance genes active against corn rootworm and lepidopteran pests (rootworm; Cry34Ab1 + Cry35Ab1, modified Cry3Bb1: lepidoptera; Cry1F, Cry1A.105, Cry2Ab2) and two genes giving tolerance to herbicides (glyphosate and glufosinate-ammonium), as a ''one-stop'' solution to pest and weed problems (Grainnet, 2007), exemplifies this gene stacking technology.

IMPROVING ON NATURE: PROTEIN ENGINEERING IN *Bt* TOXINS

Mutagenesis of Cry toxins has been used extensively in studying the mechanism of action of these proteins (Bravo et al., 2007) and has been exploited to produce novel recombinant toxins.

Domain Exchange in Three-Domain Cry Toxins

The structural similarity of all members of the family of three-domain *Bt* toxins, and the separate roles of the domains in the processes of receptor binding and channel formation, suggested that combining domains from different proteins could generate active toxins with novel specificities. Transfer of the carbohydrate-binding domain III generated a Cry1Ab-Cry1C hybrid that was highly toxic to armyworm (*S. exigua*), an insect resistant to Cry1A toxins; the presence of the Cry1Ca domain III was sufficient to confer toxicity toward *Spodoptera* (de Maagd et al., 2000). More remarkably, a hybrid Cry protein, containing domains I and III from Cry1Ba and domain II of Cry1Ia, conferred resistance to the lepidopteran pest potato tuber moth (*Phthorimaea operculella*) and to the coleopteran Colorado potato beetle (*Leptinotarsa decemlineata*) when expressed in transgenic potato (Naimov et al., 2003). The ''parental'' Cry proteins in this hybrid are lepidopteran specific, with no toxicity toward coleopterans such as the potato beetle, demonstrating the creation of a novel specificity.

Mutagenesis of Three-Domain Cry Toxins

Modification of *Bt* toxins by site-directed mutagenesis to increase toxicity toward target pests has been employed as an alternative to the ''domain swap'' approach. The key role of domain II in three-domain Cry proteins in mediating interactions with insect receptors has been exploited by mutation of amino acid residues in the loop regions of this domain. Mutation

of Cry1Ab increased its toxicity toward larvae of gypsy moth (*Lymantria dispar*) by up to 40-fold (Rajamohan et al., 1996), and similar strategies were used to increase the toxicity of Cry3A protein toward target coleopteran pests (Wu et al., 2000; see Fig. 1). The level to which rational design of toxins is possible is shown by the engineering of toxicity toward mosquito larvae into the lepidopteran-specific toxin Cry1Aa (Liu and Dean, 2006). Alternatively, a directed evolution system based on phage display technology for producing toxins with improved binding to a receptor, and thus increased toxicity, has been described (Ishikawa et al., 2007).

A current commercial transgenic maize variety with resistance to corn rootworm, MON863, expresses a modified version of the *Bt* Cry3Bb1 toxin (Vaughn et al., 2005). Unmodified Cry3Bb1 is active against a number of coleopteran species, but toxicity toward Western corn rootworm was not sufficient to give adequate protection at levels of expression achievable in maize. A large number of variants of the native Cry3Bb, incorporating a series of specific mutations that aimed to improve the channel-forming ability of the toxin, were produced and screened for activity (English et al., 2003). Mutations (see Fig. 2) were carried out to: (1) increase hydrophobicity of the protein in regions in domain I containing sheets of bound water molecules and in loop regions; (2) increase the mobility of the channel-forming helices in domain I by disrupting hydrogen-bond formation; (3) increase the mobility and flexibility of loop regions in domain I; (4) alter potential ion-pair interactions and metal-binding sites; and (5) reduce or eliminate binding to carbohydrates in the insect gut by mutation of a loop region between domains I and II. The toxicity of the protein toward corn rootworm was increased approximately 8-fold, giving a product that could be expressed at levels sufficient for adequate protection against rootworm.

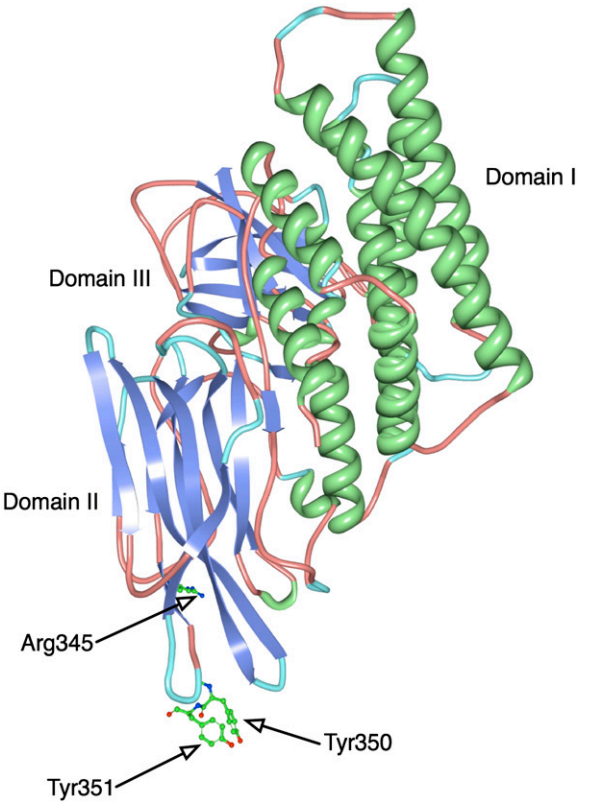

Figure 1. Engineering specificity in a three-domain Cry toxin; mutagenesis of the toxin-receptor interaction loop in domain II. Three-dimensional structure of Cry3A (1dlc; RCSB) is shown in ribbon format. Domain I (helices) is at top right, and domain II (sheet structure) is at bottom left. Domain III (carbohydrate-binding domain; sheet structure) is behind the other domains, central in this view. Residues mutated (Wu et al., 2000) to increase toxicity toward yellow mealworm (*Tenebrio molitor*), Colorado potato beetle, and cottonwood leaf beetle (*Chrysomela scripta*) are shown in ball-and stick representation. [See online article for color version of this figure.]

Recent results, showing that oligomerization of Cry toxins subsequent to binding to the cadherin "receptor" on the insect gut surface is a necessary step in the mechanism of toxicity, have led to a strategy to engineer Cry proteins to be effective against insects that have become resistant to normal toxins by receptor mutation (Soberon et al., 2007). Removal of the α-1 helix of domain I resulted in a protein that did not require to bind to cadherin to oligomerize and was toxic to resistant insects. Expression of these modified toxins in plants has yet to be attempted.

Fusion Proteins

Transformation of plants with a gene construct containing a single translationally fused coding sequence encoding two Cry proteins has been used as an alternative to separate constructs (Bohorova et al., 2001), although there is no apparent advantage over simpler methods for introducing two genes. However, addition of sequences from other proteins can be used to introduce extra functionality into Cry toxins. For example, the Gal-binding lectin domain (B-chain) from the ribosome-inactivating protein ricin was fused C terminally to domain III of Cry1Ac (Mehlo et al., 2005), giving a fusion protein that could bind to Gal residues in potential receptors in the insect, in addition to binding to *N*-acetyl galactosamine residues by domain III of the toxin. Expression of the fusion protein in transgenic maize and rice (*Oryza sativa*) plants gave resistance to larvae of stemborers (*Chilo suppressalis*) and leaf armyworm (*Spodoptera littoralis*), whereas plants expressing the unmodified Cry1Ac were susceptible to both insects. Plants expressing the fusion protein were also resistant to a hemipteran pest, the leafhopper *Cicadulina mbila*; the lectin domain may cause this effect because *Bt* toxins are not effective against hemipterans.

SELF DEFENSE: EXPLOITING PLANT DEFENSIVE PROTEINS

Engineering plants to express proteins that are end-products of the wounding response, such as proteinase

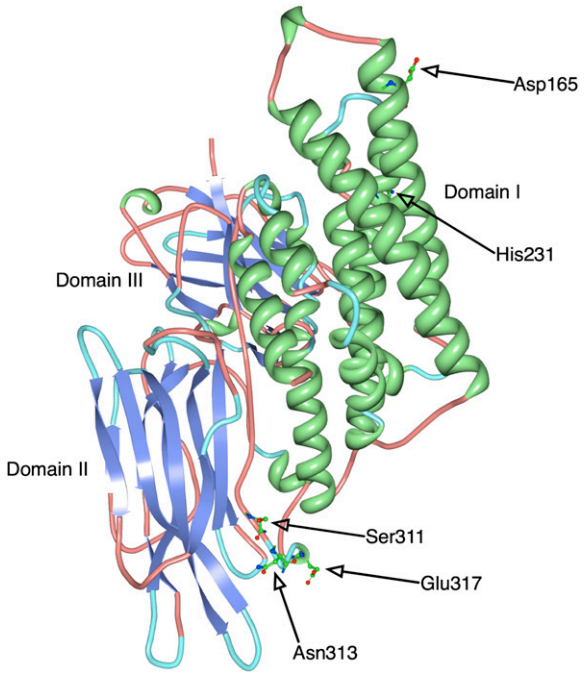

Figure 2. Engineering specificity in a three-domain Cry toxin; mutagenesis to improve channel-forming ability. Three-dimensional structure of Cry3Bb (1ji6; RCSB) is shown in ribbon format in the same view as Figure 1. Residues mutated (English et al., 2003) to increase toxicity toward corn rootworm are shown in ball-and-stick representation. Mutations are made in helices of domain I and in the region linking domains I and II. The mutation sites shown are taken from the most active toxin produced; a range of other sites for mutation were explored. [See online article for color version of this figure.]

inhibitors and polyphenol oxidase, has generally failed to give more than partial protection against insect herbivores, due to pre-adaptation by the pests. However, two examples of exploiting plant defensive proteins have shown promise in addressing specialized insect resistance problems.

Bean α-Amylase Inhibitors and Stored Product Pests

The α-amylase inhibitors from some legume seeds, which are similar to legume lectins in sequence, have been shown to be causative factors in the resistance of specific varieties of legumes to coleopteran seed weevils. The bean (*Phaseolus vulgaris*) α-amylase inhibitor gene was expressed in seeds of transgenic garden pea (*Pisum sativum*) and other grain legumes, using a strong seed-specific promoter (Shade et al., 1994). The resulting seeds contained up to 3% of the foreign protein and were resistant to stored product pests, such as larvae of bruchid beetles, and field pests, such as larvae of the pea weevil *Bruchus pisorum* (Morton et al., 2000). This strategy is directed toward coleopteran seed herbivores, which have a neutral or acidic gut pH, so the inhibitor is not inactivated, and in which starch digestion, not protein digestion, is nutritionally limiting.

Despite these results, agricultural deployment of transgenic crops expressing this α-amylase inhibitor gene has not taken place. Safety concerns have arisen as a result of the induction of systemic immunological responses in mice fed peas expressing the amylase inhibitor protein (Prescott et al., 2005), which appear to result from altered posttranslational processing in pea compared to the "natural" host (bean).

Lectins and Sap-Sucking Pests

Potential exploitation of lectin genes to confer insect resistance in transgenic plants has targeted hemipteran plant pests, which are not affected by known *Bt* toxins but have been shown to be susceptible to lectin toxicity. Expression of the Man-specific snowdrop lectin (GNA) in transgenic rice plants using constitutive or phloem-specific promoters gave plants that were partially resistant to rice brown planthopper (*Nilaparvata lugens*) and other hemipteran pests. Reductions of up to 50% in survival were observed, with reduced feeding, development, and fertility of survivors (Rao et al., 1998; Foissac et al., 2000). Concerns about possible consequences to higher animals of ingesting snowdrop lectin have limited further progress, although a recent study incorporating a 90-d feeding trial found no adverse effects resulting from consumption of transgenic rice expressing GNA by rats (Poulsen et al., 2007). Similar partial resistance to hemipterans has also been obtained by expression of a Man-specific lectin from garlic (*Allium sativum*) leaves (ASA-L) in transgenic rice (Saha et al., 2006a) and a variety of other transgenic plant species. The transgenic rice plants expressing ASA-L were shown to decrease transmission of *Rice tungro virus* by its insect vector, presumably as a result of decreased feeding by the pest (Saha et al., 2006b).

NOVEL APPROACHES: NEW INSECTICIDAL PROTEINS

Photorhabdus luminescens Insecticidal Proteins

Nematodes of *Heterorhabditis* species that contain symbiotic enterobacteria are widely used for small-scale biological control of insect pests. When nematodes enter an insect host, bacterial cells from the nematode gut are released into the insect circulatory system. Toxins secreted by the bacteria cause cell death in the insect host, leading to a lethal septicemia. *P. luminescens*, the most well-investigated bacterial species of this type, contains a large number of potentially insecticidal components (for review, see ffrench-Constant, 2007). One of the orally toxic components, toxin A, was selected for further study. The encoding gene *tcdA* was cloned and assembled into expression constructs, containing 5′ and 3′ untranslated region sequences from a tobacco osmotin gene to improve expression levels of mRNA and protein in transgenic plants. Expression of toxin A at levels >0.07% of total soluble protein in leaves of

transgenic Arabidopsis (*Arabidopsis thaliana*) plants (Liu et al., 2003) gave almost complete protection against larvae of the lepidopteran tobacco hornworm (*Manduca sexta*). Leaf extracts from these plants were also toxic to corn rootworm, showing cross-species protection. Commercial development of this technique is likely.

Cholesterol Oxidase

Bacterial cholesterol oxidase has an insecticidal activity comparable to *Bt* toxins, dependent on its enzyme activity, which is thought to promote membrane destabilization. Expression constructs containing part or all of the coding sequence of the protein, or the coding sequence fused to a chloroplast-targeting peptide, resulted in production of active enzyme in transgenic tobacco (Corbin et al., 2001). However, phenotypic abnormalities were observed in transgenic plants unless the enzyme was localized in chloroplasts, possibly as a result of interference with steroidal signaling pathways. Leaf tissue from all transgenic plants was toxic to boll weevil larvae. The cholesterol oxidase gene appears to be an obvious candidate for introduction into the chloroplast genome (see above) rather than the plant nuclear genome, which would avoid potential problems caused by enzyme activity in the cytoplasm.

Avidin as an Insecticidal Protein

Avidin has a strong insecticidal effect on many insects, although susceptibility varies widely between different insect species (apparently based on biotin requirements). Expression of avidin in transgenic maize initially aimed to produce the protein as a high-value product, but maize seed containing more than 0.1% avidin (of total protein) was fully resistant to larvae of three different coleopteran storage pests (Kramer et al., 2000). The protein has also been expressed in other transgenic plants to confer pest resistance. Targeting of the foreign protein away from the cell cytoplasm (e.g. using targeting sequences from potato proteinase inhibitors; Murray et al., 2002) is important to avoid developmental abnormalities in the plants. No further development of this promising method has been reported.

NOVEL APPROACHES: EXPLOITING SECONDARY METABOLISM

Engineering Secondary Metabolism of Plant Defensive Compounds

The availability of genes encoding the biosynthetic enzymes of secondary metabolism has made transfer of biosynthetic pathways between plants feasible. Genes encoding two Cyt P450 oxidases and a UDP-glycosyltransferase from sorghum (*Sorghum bicolor*) have been transferred to Arabidopsis (Tattersall et al., 2001), resulting in the production of the cyanogenic glycoside dhurrin from Tyr (Kristensen et al., 2005). The resulting plants produced hydrogen cyanide on tissue damage and showed enhanced resistance to attack by the flea beetle *Phyllotreta nemorum*, a specialist feeder on crucifers. Other secondary metabolites that have been produced in transgenic plants include the alkaloid caffeine (in tobacco; by the introduction of three genes encoding *N*-methyl transferases; Kim et al., 2006).

Engineering Secondary Metabolism of Volatile Communication Compounds

Engineering volatiles emitted by plants offers possibilities for new methods of crop protection. Volatile composition has been altered in tobacco by RNA interference (RNAi)-mediated suppression of a *cytP450* oxidase gene expressed in trichomes, and in Arabidopsis by constitutive overexpression of a plastid dual linalool/nerolidol synthase (Wang et al., 2001; Aharoni et al., 2003). The transgenic plants deterred aphid colonization but were not wholly resistant. Volatiles can also be used as attractants for natural enemies of pests; for example, Arabidopsis plants transformed with the maize terpene synthase gene *TPS10* emitted the sesquiterpene volatiles normally produced in maize and attracted parasitoid wasps that attack maize pests (Schnee et al., 2006). Volatiles used by insects to communicate with each other can also be exploited; the sesquiterpene (*E*)-β-farnesene is an alarm pheromone in aphids and attracts aphid predators and parasitoids (Beale et al., 2006). When Arabidopsis was transformed with an (*E*)-β-farnesene synthase gene from mint (*Mentha* $\times$ *piperita*), the resulting plants showed significant levels of aphid deterrence in choice experiments and were attractive to the aphid parasitoid *Diaeretiella rapae*.

RNAi

Disrupting gene function by the use of RNAi is a well-established technique in insect genetics based on delivery by injection into insect cells or tissues. The observation that RNAi could also be effective in reducing gene expression, measured by mRNA level, when fed to insects (Turner et al., 2006) has led to two recent articles in which transgenic plants producing double-stranded RNAs (dsRNAs) are shown to exhibit partial resistance to insect pests. Transgenic maize producing dsRNA directed against V-type ATPase of corn rootworm showed suppression of mRNA in the insect and reduction in feeding damage compared to controls (Baum et al., 2007). Similarly, transgenic tobacco and Arabidopsis plant material expressing dsRNA directed against a detoxification enzyme (Cyt P450 gene CYP6AE14) for gossypol in cotton bollworm caused the insect to become more sensitive to gossypol in the diet (Mao et al., 2007). This approach holds great promise for future development.

PRACTICAL SUCCESS, PUBLIC RELATIONS FAILURE? THE PROSPECTS FOR ENGINEERED INSECT-RESISTANT CROPS IN THE REAL WORLD

The production of transgenic insect-resistant plants, and their continuing development, has been a major scientific success, mirrored by the practical success of a limited number of pest-resistant genetically modified crops in some countries. However, these successes must be set against the failure to make this technology more widely available. In some developed countries, this has been a result of vocal opposition to plant genetic engineering technology itself; but in many instances, in both developed and developing countries, it is more a case of potential economic returns not being sufficient to make the introduction of engineered crop varieties commercially viable. There is a need to reconsider regulatory systems for release of transgenic crops. In most cases, these were set up with good intentions, but the end result in developed countries has been to make commercialization of transgenic crops difficult and expensive, so that only very large companies can afford to carry products through and then only when the projected returns are very large. Under these circumstances, it is not surprising that campaigns against genetically modified crops have been so successful, no matter how ill-founded their scientific basis may be.

In the developed world, the economic consequences to the public of the failure to adopt transgenic insect-resistant plants more widely have been seen as marginal, and the potential environmental benefits are discounted (even when clearly demonstrated; Nature Biotechnology Editorial, 2007). The economic and environmental consequences for the developing world, where significant benefits to the lives of farmers could be obtained from this technology (Huang et al., 2005), are more serious. Here, access and implementation are being hindered by the actions of companies and organizations in developed countries. A change in attitude by governments, nongovernmental organizations, and the public at large in some countries is needed for insect-resistant transgenic crops to be able to fulfill their promise to all the world's population, not just to the few.

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