

Review

Engineering insect-resistant crops: A review

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Insect pests cause significant damage to crops world-wide. This is despite integrated pest management strategies combining such control measures as chemical control, use of resistant varieties and other measures. Other control measures such as use of genetically modified crops are being adopted. Transgenic crops engineered for enhanced levels of resistance to insect pests have the potential to offer large benefits to agriculture not only through enhanced crop protection, but also from a reduction in the number of insecticide treatments required compared to conventional cropping methods. Insect resistant crops expressing *Bacillus thuringiensis* (*Bt*) delta-endotoxins are currently being grown in many regions of the world. *B. thuringiensis* is a gram positive, spore-forming bacterium that produces crystalline inclusion bodies during sporulation. These contain insecticidal delta endotoxins, also known as insecticidal crystal proteins. There are two groups of insecticidal crystal proteins (ICP): Cry (crystal delta endotoxins) and Cyt (cytolytic). The specificities of the different insecticidal crystal proteins determine their subsequent toxicity. Cry toxins are classified by their primary amino acid sequence and more than 500 different Cry gene sequences have been classified into 67 groups (Cry1 to Cry67). They are globular molecules composed of three distinct functional domains connected by a short conserved sequence. Two major types of receptors have been identified: transmembrane proteins, such as cadherins, and proteins anchored to the membrane such as the glycosylphosphatidylinositol (GPI)-anchored proteins that have been proposed to be involved in the action of Cry toxins. The continued use of transgenic crops is threatened by the evolution of resistance in insect populations. It is against this background that research work targeting other candidate genes such as proteinase inhibitors, lectins and secondary metabolites is gaining momentum.

Key words: Transgenic crops, *Bacillus thuringiensis*, insecticidal crystal proteins, glycosylphosphatidylinositol (GPI)-anchored.

INTRODUCTION

Losses due to insects have increased over the last two decades. This is despite integrated pest management strategies combining such control measures as chemical control, use of resistant varieties and other measures (Duck and Evola, 1997). Insects have also evolved resistance to almost all the pesticides in use today. It is against this background that other control measures such as use of genetically modified crops are being adopted.

Transgenic crops engineered for enhanced levels of resistance to insect pests have the potential to offer large benefits to agriculture (Ferry et al, 2003), not only through enhanced crop protection, but also from a reduction in the number of insecticide treatments required compared to conventional cropping methods (James, 2005).

However, despite the advantages that such crops offer, major concerns relating to their use are routinely voiced:

potential toxicity to humans, effect on non-target organisms, and potential deleterious environmental effects. GM crops are subjected to a robust and rigorous safety assessment starting with a comparative analysis between the chemical composition and nutritional value of GM crops and conventional varieties (Phipps, 2009). To date, numerous studies have been carried out with dairy cows, pigs, poultry and fish, all of which have failed to detect the presence of 'transgenic' DNA fragments with genetic integrity or novel proteins in any animal derived food. Furthermore, no evidence has been found to suggest that food derived from animals fed GM feeds is anything other than as safe as that produced by conventional feed ingredients. Also, there has been no evidence to suggest that any commercial GM crops are deleterious to humans.

To ensure that transgenic crops, including those expressing genes for enhanced resistance to pests, do not confer deleterious effects on non-target organisms, including beneficial insects, such crops undergo a tiered risk assessment (Romeis, 2009). Of the numerous studies carried out to date, including both lab-based and field trials, only one or two have suggested potential deleterious effects, with the vast majority demonstrating no toxic effects; interestingly there is evidence of increased biodiversity where GM crops are grown, this being a consequence of decreased pesticide usage.

Transgenic crops were grown on 160 million hectares of land in 2011 (Figure 1), with insect resistant crop varieties representing approximately 15% of this area (James, 2012). The global market value of biotech crops was estimated at US\$ 13.2 billion. Insect resistant crops expressing *Bacillus thuringiensis* (*Bt*) delta-endotoxins are currently being grown in many regions of the world.

***Bacillus thuringiensis* (*Bt*) toxins**

B. thuringiensis is a soil bacterium first discovered by Ishawata in Japan in 1901 and then in Germany in 1911 by Berliner (Baum et al., 1999). It is a Gram positive, spore-forming bacterium that produces crystalline inclusion bodies during sporulation (Ferré et al., 1991; Vadlamudi et al., 1993; Valaintis et al., 1997; Estela et al., 2004; Flannagan et al., 2005). These contain insecticidal delta endotoxins, also known as insecticidal crystal proteins (Cry); they accumulate in the mother cell and can account for 20 to 30% of the dry weight (Schnepf et al., 1998). There are two groups of insecticidal crystal proteins (ICP): Cry (Crystal delta endotoxins) and Cyt (Cytolytic).

The specificities of the different insecticidal crystal proteins determine their subsequent toxicity. Different strains of *Bt* produce different Cry proteins and these vary in their toxicity towards different insects: Cry I are specific for Lepidoptera; Cry II are specific for Lepidoptera and Diptera; Cry III are specific for Coleoptera and Cry IV

are specific for Diptera (Hofte and Whiteley, 1989). The Cry 1 subclass is in the range of 120-140kDa in size (Masson, 2002).

Structure of *Bt* toxin

The tertiary structure of seven different Cry proteins, Cry 1Aa, Cry2Aa, Cry3AA, Cry3Bb, Cry4Aa and Cry8Ea have been determined by X-ray crystallography (Li et al., 1991; Grochulski et al., 1995; Galitsky et al., 2001; Morse et al., 2001; Boonserm et al., 2005; Boonserm et al., 2006; Guo et al., 2009).-These structures display a high degree of similarity with a three-domain organisation (Figure 2) and this suggests a similar mode of action of the Cry protein family even though they show very low amino acid sequence similarity.

Cry toxins are classified by their primary amino acid sequence and more than 500 different Cry gene sequences have been classified into 67 groups (Cry1-Cry67). They are globular molecules composed of three distinct functional domains connected by a short conserved sequence.

The N-terminal domain (Domain I) is a bundle of seven α -helices in which the central helix α -5 is hydrophobic and is encircled by six other amphipathic helices (Pigott and Ellar, 2007). This helical domain is similar in structure to the pore-forming domain of colicin (Parker et al., 1989) and is therefore thought to be involved in pore formation (Li et al., 1991).

Domain II is made up of three anti-parallel beta sheets packed together to form a beta-prism (Li et al., 1991) with exposed loop regions. Domain II is thought to play a role in receptor binding and specificity because of the variability of its structure (Pigott and Ellar, 2007). Domain II shares structural similarity with several carbohydrate-binding proteins such as vitelline and lectin jacalin (de Maagd et al., 2003).

Domain III is a sandwich of two twisted anti-parallel beta sheets (Schnepf et al., 1998). These sheets are made up of five strands and the outer sheet faces the solvent and the inner one packing against Domain II (Pigott and Ellar, 2007). It plays a role in insect specificity and receptor binding. The exposed regions in domains II and III are involved in receptor binding (Bravo et al, 2005).

Domain III, shares structural similarities with other carbohydrate binding proteins such as cellulose binding domain of 1,4- β -glucanase C, galactose oxidase sialidase, β -glucuronidase, carbohydrate binding domain of xylanase U and β -galactosidase (de Maagd et al., 2003).

Receptors for *Bt* toxins

Two major types of receptors have been identified: trans-membrane proteins, such as cadherins, and proteins anchored to the membrane such as the Glycosyl phosphatidyl inositol (GPI)-anchored proteins that have been

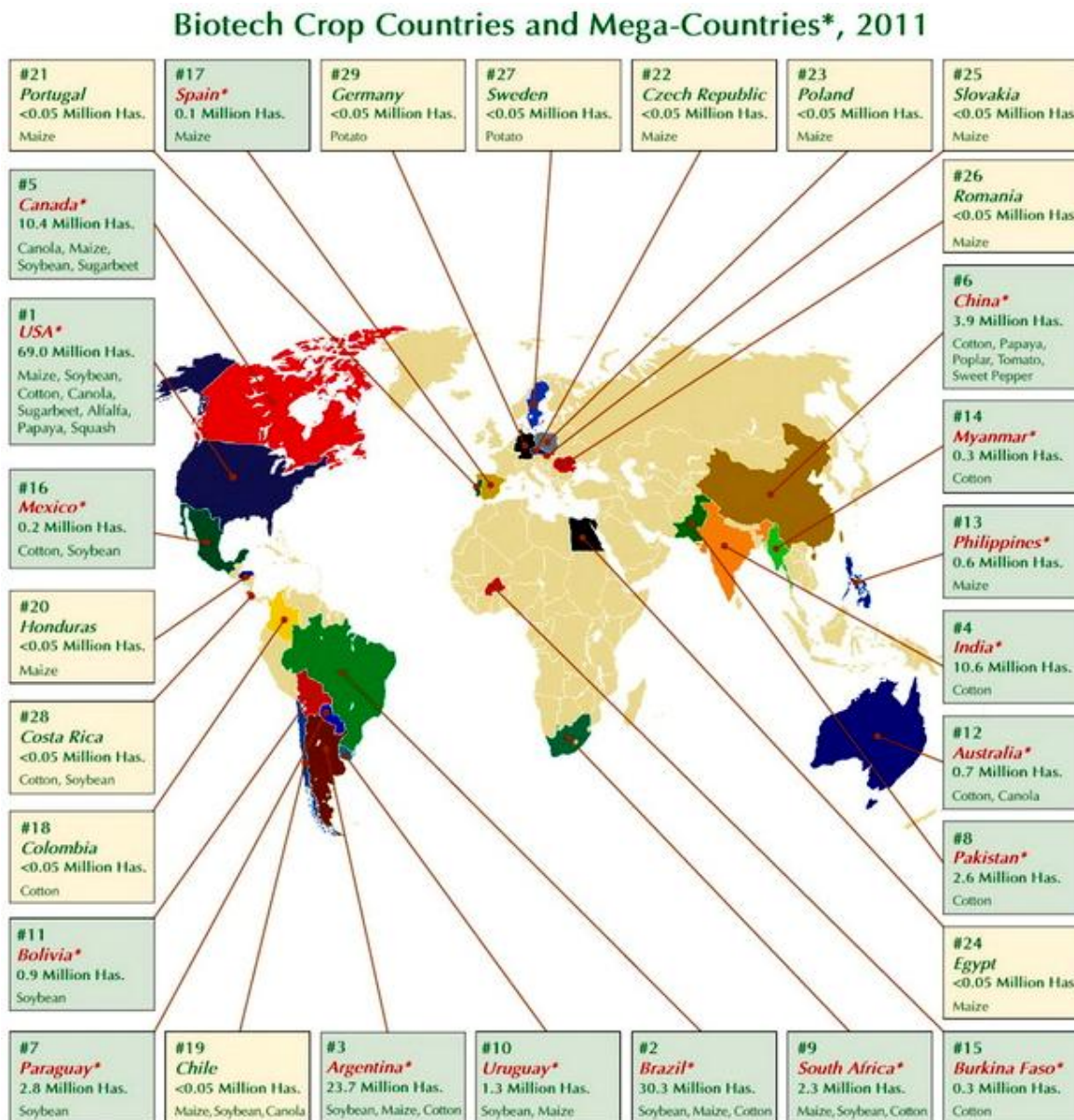


Figure 1. Global map of biotech crop countries and mega-countries in 2011. Source: Clive James (2011).

proposed to be involved in the action of Cry toxins (Gomez et al, 2007).

Cadherins

Cadherins are synthesised as a precursor polypeptide which requires post-translational modifications to form a protein of between 723 and 748 amino acid long. They are composed of an ectodomain formed by 11 to 12 cadherin repeats, a transmembrane domain and an intracellular domain (Bel and Escrache, 2006). Cry 1A

toxins bind to cadherin proteins of at least six lepidopteran species, *Manduca sexta*, *Bombyx mori*, *Heliothis virescens*, *Helicoverpa armigera*, *Pectinophora gossypiella* and *Ostrinia nubilalis* (Pigott and Ellar 2007).

They typically consist of five cadherin repeats (Angst et al., 2001) but as many as 34 may be present (Dunne et al., 1995). Three regions in CADR proteins have been shown to interact with three domain II loop regions. Cry1Ab loop 2 interacts with CADR residues ⁸⁶⁵NITIHITDNN⁸⁷⁵ located in repeat 7, loops a-8 and 2 interacts with CADR residues ¹³³¹IPLPASILTVTV¹³⁴²

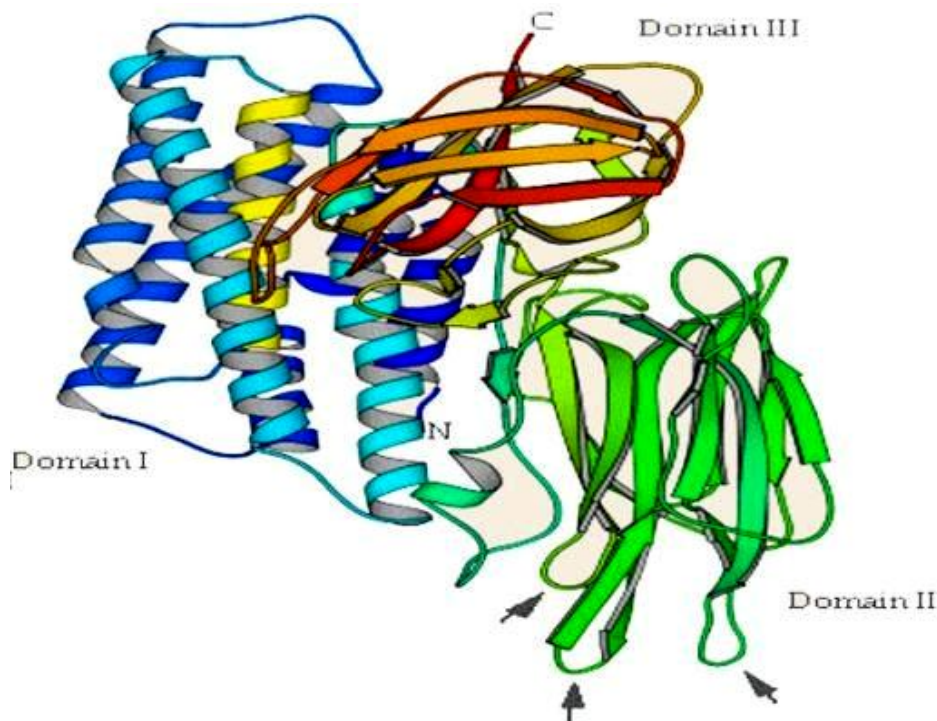


Figure 2. Putative structure of the 3-domain Cry proteins.

located in repeat 11. A third Cry1A binding region was located in CADR in the repeat 12 (Gomez et al., 2007).

GPI-anchored receptors: Amino peptidase N (APN) and alkaline phosphatase (ALP)

The APN and ALP in *M. sexta* and *H. virescens* have been shown to be located in lipid rafts. The interaction of pore-forming toxins with lipid rafts is said to result in toxin internalization, signal transduction and cellular response (Zhuang et al., 2002; Bravo et al., 2004). The first Cry 1A toxin binding protein that was described was an APN protein in *Manduca sexta*. Other GPI-anchored APNs have been recognised as Cry toxins receptors in different Lepidoptera such as *Heliothis virescens*, *Spodoptera litura*, *Helicoverpa armigera*, *Bombyx. mori*, *Lymantria dispar*, *Plutela xylostalla* and in dipteran *An quadrimaculatus* and *A. aegypti*.

Alkaline phosphatases are mainly localised in microvilli of the columnar cells and of insect epithelial cells (Eguchi, 1995). They are divided into two groups: soluble (s-ALP) and membrane bound (m-ALP) (Eguchi, 1995). The s-ALP is found exclusively in the cavity of goblet cells and in the apical region of the midgut, whereas, m-ALP is localised in the brush border membrane of columnar cells. Alkaline phosphatase has been implicated as a receptor for Cry toxins in some species such as *M. sexta* (Sangadala et al., 1994) and *H. virescens* (Jurat-Fuentes and Adang, 2004).

Mode of action of *Bt* toxins

Currently two main models have been proposed for the mode of action of *Bt* the Bravo model (pore formation model) and the Zhang model (signal transduction model).

The Bravo Model is an updated version of the model originally proposed by Knowles and Ellar (Pigott and Ellar, 2007) (Figure 3). According to this model, both cadherin and APN are required for Cry1A toxicity towards *M. sexta* (Bravo et al., 2004). The model proposes that the activated toxin binds to cadherin, BT-R1 and the toxin undergoes a conformational change resulting in cleavage of helix alpha-1 by membrane-bound proteases (Pigott and Ellar, 2007). The toxin oligomerises and binds to APN. The toxin oligomer inserts into lipid membranes where it forms pores and this leads to the death of the larva (Bravo et al., 2007). Studies show that insecticidal proteins cause histopathological symptoms on the midgut (George et al., 2012). These include vacuolation of the cytoplasm and unzipping of the septate junctions binding columnar cells (George et al., 2012). The Zhang model proposes that binding of Cry1 to cadherin triggers a signal that involves the stimulation of a guanine nucleotide binding protein (G protein) and adenylate cyclase to increase cAMP. This results in the activation of protein kinase A leading ultimately to cell death (Zhang et al., 2006).

A direct correlation between toxicity and receptor binding has been observed in most studies (Hofmann et al., 1988; Lu et al., 1994; Bravo et al., 1992). However,

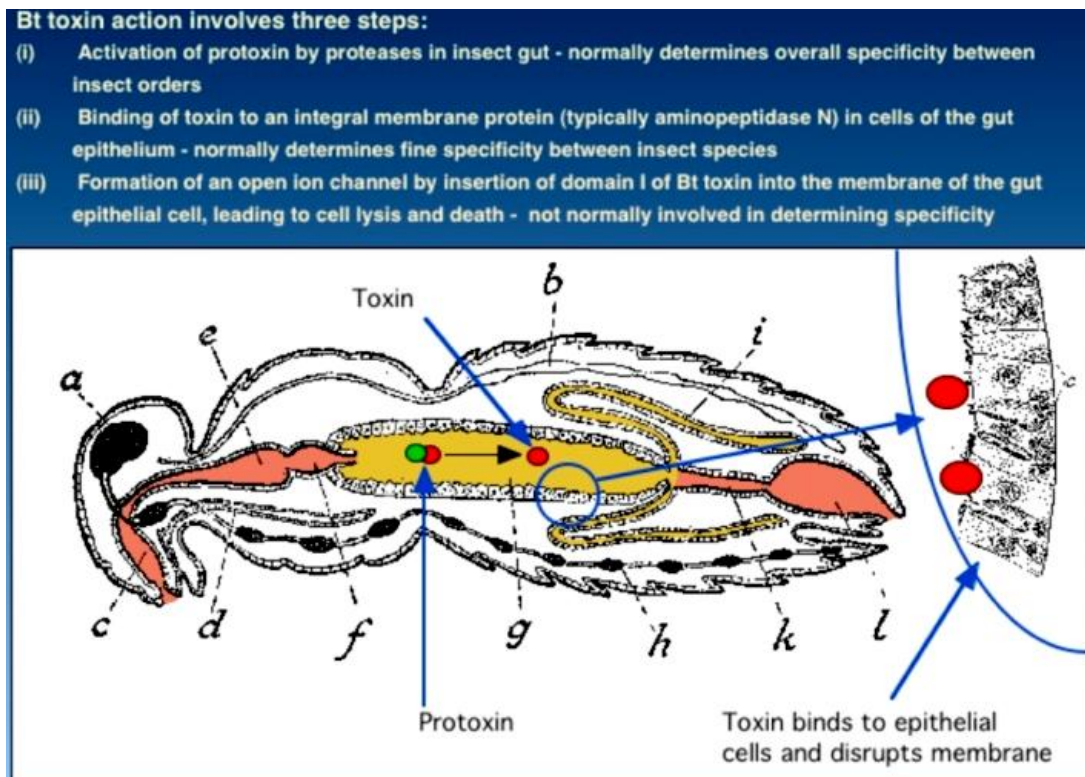


Figure 3. Mechanism of action of *Bt* toxin (Courtesy of J.A.Gatehouse).

there have been some reported exceptions (Garczynski et al., 1991; Wolfersbeger et al., 1990) but no insecticidal crystal protein has been found to be toxic without prior binding to the brush border membrane of the midgut epithelial cells (Bravo, 1992).

POSSIBLE MECHANISMS OF RESISTANCE TO *Bt*

The continued use of transgenic crops is threatened by the evolution of resistance in insect populations (Bravo and Soberon, 2008). The molecular basis of resistance to the *Bt* in insects is not well understood. However, this information is important for developing strategies to cope with the evolution of pest resistance (Merroquin et al., 2000). Mode 1 is the most common type of resistance in insects. It is characterised by reduced binding of *Bt* toxins to target sites of the insect midgut membrane (Tabashnik et al., 1998). In *H. virescens* and *Pectinophora gossypiella* mutations affecting Cry1A binding to the midgut, cadherin protein was linked to laboratory selected Mode 1 resistance in these species. It has been reported that Cry1Ac resistance in tobacco budworm, *H. virescens* is linked to cadherin encoding genes but not to genes encoding aminopeptidases (Gahan et al., 2001). Loss of a proteinase in Indian meal moth was said to confer resistance to Cry1Ab (Herero et al., 2001) in this insect. In susceptible insects, this proteinase enzyme cleaves

the Cry1Ab protoxin to form an active toxin. It has been reported that mutations in five different genes conferred resistance to Cry 5B in *Caenorhabditis elegans* (Merroquin et al., 2000). In all these genes, none resembled genes for aminopeptidase or cadherins.

In field conditions, three lepidopteran insects have evolved resistance to formulated *Bt* products, *Plodia interpunctella*, *Plutella xylostella* and *Trichoplusia ni* [McGaughey, 1985; Tabashnik, 1994; Janmaat and Myers, 2003]. It was reported that *Helicoverpa zea* has evolved resistance to *Bt* expressing cotton in United States (Tabashnik et al., 2008). This was the first reported case of resistance to transgenic crops in the field. Other cases of resistance to *Bt* crops have also been documented; *S. frugiperda* to *Bt*-corn expressing Cry1F in Puerto Rico, *Busseola fusca* to *Bt*-corn expressing Cry1Ab in South Africa and *P. gossypiella* to *Bt*-cotton expressing Cry1ac in India (Gill et al., 2011). Thus, resistance management is a key component of any strategy that utilizes transgenic crops.

RESISTANCE MANAGEMENT

Gene stacking/pyramiding

Gene stacking is when two or more toxins with different modes of action are produced in the same plant (Bravo and Soberon, 2008). This strategy has been successful in

a number of crops such as cotton and maize. Expressing more than one *Bt* cry gene in plants affords the plant more protection against a wider range of pests (Gatehouse, 2008). Cotton plants expressing Cry1Ac and Cry2Ab were found to be more toxic to bollworms (*H. zea*) and armyworms (*Spodoptera frugiperda* and *Spodoptera exigua*) than cotton expressing Cry1Ac alone in laboratory trials (Stewart et al., 2001).

Gene stacking is also beneficial in preventing evolution of resistance to toxin activity in the target pest(s) (Gatehouse, 2008). However, it has recently been shown that pests can acquire resistance to multiple toxins. Gahan et al. (2005) reported that *H. virescens* evolved resistance to Cry1Ac and Cry2Aa. To counter evolution of resistance in insects, crops expressing multiple transgenes are being developed. Transgenic maize expressing six insect resistant genes has been developed (Gatehouse, 2008). The genes afford resistance against corn rootworm (Cry34Ab1+Cry35Ab1, modified Cry3Bb1) and lepidopteran (Cry1F, Cry1A.105, Cry2Ab2) pests. The plant also expresses two genes giving tolerance to glyphosate and glufosinate ammonium herbicides.

Use of Refugia

The use of refugia is another strategy that is used to manage or delay resistance to *Bt* crops. Under this strategy, a non-*Bt* crop is planted in areas of land adjacent to the *Bt* crop and this serves as a reservoir for susceptible insects. The few resistant individuals from *Bt* crops will mate with susceptible insects and this results in a low rate of resistance. The success of this strategy depends on a number of factors: the resistant trait must be recessive, there must be random mating, and there must be no insecticidal action in the refugia.

OTHER CANDIDATES GENES

Proteinase inhibitors

Protease inhibitors are categorised on the basis of their specificity. Four mechanistic classes have been identified: inhibitors of serine, aspartyl, cysteine and metallo proteases. Serine proteinase inhibitors have anti-nutritional effects against several lepidopteran insect species since they inhibit protease activity and consequently reduce protein digestion (Shulke and Murdock, 1983; Applebaum, 1985). This results in nitrogen deficiency, and in an attempt to compensate, the insect over-produces digestive proteases (themselves proteins) leading to further loss of amino acids (Shulke and Murdock, 1983). Characterisation of the proteolytic activity in the larval midgut of the sub-Saharan African pest *B. fusca* demonstrated that this lepidopteran stem borer utilises serine proteases for protein digestion (George et al., 2008).

Alpha amylase inhibitors

Alpha-amylases (α -1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a family of endo-amylases that catalyse the-hydrolysis of α -D-(1 \rightarrow 4)-glucan linkages in starch components, glycogen and other carbohydrates. The enzymes play a key role in carbohydrate metabolism of microorganisms, plants and animals (Franco et al., 2002). Several insects, especially those similar to the seed weevils that feed on starchy seeds during larval and/or adult stages, depend on their α -amylases for survival (Franco et al., 2002). Alpha-Amylase inhibitors are therefore, attractive candidates for the control of seed weevils because they are highly dependent on starch as an energy source (Franco et al., 2002).

The first practical demonstration involving α -amylase inhibitors used α -A11, which specifically inhibits the α -amylases of the three bruchis; the pea weevil, *Bruchus pisorum*, the cowpea weevil and the azuki bean weevil (Franco et al., 2002). Azuki bean plants expressing α -A11 were found to be completely resistant to azuki bean weevil (Ishimoto et al, 1996).

Lectins

Lectins are a group of non-immunogenic proteins possessing at least one non-catalytic domain that binds reversibly to specific mono-or oligosaccharide (Peumans and Van Damme, 1996). Mannaose-binding lectins are widely distributed in the higher plants and are believed to play a role in recognition of high-mannose type glycans of foreign micro-organisms or plant predators (Barre et al., 2001). A gene encoding the mannose specific lectin from snowdrop (*Galanthus nivalis*; GNA) expressed in tobacco has shown enhanced resistance to peach potato aphid (*M. persicae*) and a pea lectin in tobacco has shown enhanced resistance to *H. virescens* (Boulter et al., 1990). Greater insecticidal activity has also been observed in chitin-binding lectins and the lectin gene of wheat germ and the common bean (Sarma et al., 2004).

Transgenic rice shoots with GNA have shown resistance to brown plant hopper (BPH) (*Nilaparvata lugens*) and the green plant hopper (GLH) (*Nephotettix virescens*) (Yang et al., 1998) and potato leafhopper (*Empoasca fabae*). Partial resistance to hemipterans has also been obtained by expression of Man-specific lectin from garlic (*Allium sativum*) leaves in transgenic rice (Saha et al, 2006). However, concerns about the possible consequences to higher plants of ingesting snowdrop lectin have limited further progress (Gatehouse, 2008).

Secondary metabolites

Plants use a number of defence strategies against biotic attackers (Walling, 2000; Kessler and Baldwin, 2001). These defence strategies can be direct or indirect defences (Lawrance and Novak, 2004). Direct defences are

compounds that interfere with insect feeding and nutrition (Kessler and Baldwin, 2002). These include proteinase inhibitors that inactivate digestive enzymes. Maize lines with resistance to several lepidopteran insects have been shown to mobilize a 33kDa cysteine protease when attacked by *Spodoptera frugiperda* (Pechan et al., 2000). Indirect defences include the release of plant volatile organic compounds that attract natural enemies of herbivorous insects (Roda et al., 2004).

Mattiacci et al. (1995) showed that an enzymatic elicitor, β -glucosidase, isolated from *Pieris brassicae* regurgitant, elicits the release of parasitoid-attacking volatiles from cabbage leaves. The regurgitant of *Spodoptera exigua* was shown to elicit the release of parasitoid attracting volatile organic compounds from corn (Alborn et al., 1997). The elicitor was shown to be N-(17-hydroxylinolenoyl)-L-gutamine, a non-enzymatic elicitor. Most of these elicitors are from the insect and are fatty acids amino acid conjugates (FAC) and they arise from the insect's alimentary canal (Spiteller et al., 2000; Lait et al., 2003). Variation in the amount of FACs in the insect regurgitant has been reported from different species, different larval stages and individual collections of regurgitant from the same species (Pohnert et al., 1999; Mori et al., 2001, Alborn et al., 2003). There are other elicitors that originate from the insect salivary glands (Roda et al., 2004).

The plant's response to attack from different insects can also differ. It has been shown that volatile organic compounds VOCs elicited by attack from *H. zea* and *H. virescens* larvae differed (De Moraes et al., 1998).

Genes encoding two Cyt P450 oxidases and a UDP-glycosyl transferase from sorghum 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 plant produced hydrogen cyanide on tissue damage and showed enhanced resistance to attack by flea beetle (*Phyllotreta nemorum*) (Gatehouse, 2008). Alkaloid caffeine has been produced in tobacco plants by the introduction of three genes encoding N-methyl transferase (Kim et al., 2006).

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