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Biotechnological Approaches for the Control of Insect Pests in Crop Plants

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1. Introduction

Each year billions of dollars are spent worldwide on insect control in agriculture [1]. Despite this expenditure, up to 40% of a crop can be lost to insect damage, particularly in developing countries [2]. Some of the most damaging insect species belong to the Lepidoptera, the second largest insect order comprised of moths and butterflies. The larval stage of moths cause major damage to an array of economically valuable crops including cotton, tobacco, tomato, corn, sorghum, lucerne, sunflower, pulses, and wheat [3]. Until recently, broad spectrum chemical insecticides have been the primary control agent for agricultural pests, with about 40% targeted to the control of lepidopteran insects [4]. Over the years the widespread use of pesticides has led to pesticide resistant insects, a reduction in beneficial insect populations and harmful effects to humans and the environment [5-8]. These problems have led researchers to develop different insect control strategies using both synthetic and natural molecules that are more environmentally friendly.

One such approach has been the use of transgenic plants expressing plant defence molecules. Genetic modification can potentially provide a much larger array of novel insecticidal genes that are otherwise beyond the scope of conventional breeding. The first transgenic plant that expressed an insecticidal gene was produced in 1987. The transgenic tobacco plant produced cowpea trypsin inhibitor at levels of up to 1% of the soluble protein and had enhanced protection against the lepidopteran pest *Heliothis virescens* [9,10]. The gene encoding the cowpea trypsin inhibitor was subsequently transferred into rice [11] and potato [12,13], but did not provide sustainable insect protection and was thus not commercially viable. Commercial development of insecticidal genes has focused on the *Bacillus thuringiensis* (Bt) toxins [14,15]. In 1987, genes encoding the Bt endotoxins were also transformed into tobacco and tomato plants [16-18]. Since the commercialisation of biotech crops in 1996, farmers have adopted the technology at such a dramatic rate, that in 2011, 16.7

million farmers in 29 counties planted 160 million hectares of the biotech crops. This has led to a reduction in chemical pesticide use of 443 million Kg and an additional financial gain for farmers of US \$78 billion in the last 15 years [19]. In India alone, Bt-cotton has increased cotton yields by up to 60%, and has reduced insecticide sprays by around half. This in turn has led to an income increase of up to US \$11.9 billion per annum [19]. The reliance of a worldwide industry on one insect resistance trait has led to real concerns about the development of Bt-resistant insects [20], especially since at least four cases of field based resistance have already been documented [21-23]. This in turn has led to a search for new insecticidal proteins and their encoding genes that have commercial potential for plant protection [8,24]. They include α -amylase inhibitors [25,26], vegetative insecticidal protein [27,28], chitinases [29] and protease inhibitors [30,31], as well as several other proteins directed to targets in the insect gut (Table 1).

Transgene	Source and Mode of Action	Example of use
<i>Bacillus thuringiensis</i> (Bt) endotoxin	See section “The <i>Bacillus thuringiensis</i> endotoxin”	See section “The <i>Bacillus thuringiensis</i> endotoxin”
Vegetative insecticidal protein (VIP)	VIPs are produced by <i>Bacillus cereus</i> and <i>Bacillus thuringiensis</i> . They have similar activity to endotoxins from Bt. Vip1/Vip2 are toxic to coleopteran insects and Vip3 is toxic to lepidopteran insects [32].	VIP was highly toxic to <i>Agrotis</i> and <i>Spodoptera</i> species. VIP induced gut paralysis, complete lysis of the gut epithelial cells and resulted in larval mortality [33].
		<i>Agrotis ipsilon</i> and <i>Spodoptera frugiperda</i> larvae suffered gut paralysis, disruption of midgut epithelial cells and mortality on Vip3A [34].
		Vip3A was toxic to <i>A. ipsilon</i> and <i>S. frugiperda</i> . Larvae of <i>Ostrinia nubilalis</i> and <i>Danaus plexippus</i> were insensitive [35].
		Vip3Aa14 was toxic to <i>Spodoptera litura</i> and <i>Plutella xylostella</i> . Larvae of <i>Helicoverpa armigera</i> and <i>Pieris brassicae</i> were insensitive [27].
		VIP3Ac1 had insecticidal activity against larvae of <i>S. frugiperda</i> , <i>Helicoverpa zea</i> and <i>Trichoplusia ni</i> , but low activity against <i>Bombyx mori</i> and <i>O. nubilalis</i> . The chimeric protein Vip3AcAa was insecticidal to <i>O. nubilalis</i> [28].
		Vip3LB resulted in growth inhibition of <i>Spodoptera littoralis</i> when incorporated into a semi solid artificial diet [36].

Transgene	Source and Mode of Action	Example of use
Biotin binding proteins (avidin and streptavidin)	Biotin is an essential vitamin for insects. It functions as a covalently-bound cofactor in various carboxylases, which have major roles in gluconeogenesis, lipogenesis, amino acid and fatty acid catabolism, and the citric acid cycle.	Avidin and streptavidin increased mortality in four Lepidoptera; <i>Epiphyas postvittana</i> , <i>Planotortrix octo</i> , <i>Ctenopseustis obliquana</i> and <i>Phthorimaea operculella</i> when incorporated into artificial diets [37].
	Avidin is a water-soluble tetrameric glycoprotein from chicken egg, which binds strongly to biotin. Streptavidin is a homologous protein found in the culture supernatant of <i>Streptomyces avidinii</i> .	Transgenic plants with leaves expressing avidin in the vacuole halted growth and caused mortality in <i>H. armigera</i> and <i>S. litura</i> larvae [38].
		Transgenic tobacco plants expressing either avidin or streptavidin increased mortality of the potato tuber moth (<i>P. operculella</i>). Similarly, transgenic apple expressing either avidin or streptavidin increased mortality and decreased growth of the lightbrown apple moth (<i>E. postvittana</i>) [39].
		Transgenic tobacco expressing avidin reduced <i>S. litura</i> larval mass [40].
		Transgenic tobacco expressing three variants of biotin binding proteins in the vacuole increased mortality of <i>P. operculella</i> larvae [41].
Chitinase (enzyme)	Chitinase catalyses the hydrolysis of chitin, which is one of the vital components of the lining of the digestive tract in insects and is not present in plant and higher animals.	Transgenic tobacco plants expressing <i>M. sexta</i> chitinase caused a reduction in survival and growth of <i>H. virescens</i> , but not <i>M. sexta</i> larvae [42].
		<i>Lacanobia oleracea</i> larvae exposed to diet containing recombinant <i>L. oleracea</i> chitinase had a reduction in weight gain and consumption compared to control-fed larvae [43].
		Transgenic rapeseed (<i>Brassica napus</i>) expressing <i>M. sexta</i> chitinase and scorpion insect toxin increased mortality and reduced growth of <i>Plutella maculipennis</i> [44].

Transgene	Source and Mode of Action	Example of use
		Oral injection of <i>B. mori</i> chitinase (<i>Bm</i> -CHI) caused high mortality in Japanese pine beetle, <i>Monochamus alternates</i> (Coleoptera). The peritrophic membrane chitin was degraded by <i>Bm</i> -CHI, but the midgut epithelium was not affected [29].
Cholesterol oxidase (enzyme)	Cholesterol oxidase is a bacterial enzyme that catalyzes the oxidation of cholesterol and other 3-hydroxysterols, resulting in production of the corresponding 3-hydroxysterols and hydrogen peroxide. Functions by damaging midgut membranes.	Cholesterol oxidase from <i>Streptomyces</i> caused stunting of <i>H. virescens</i> , <i>H. zea</i> and <i>Pectinophora gossypiella</i> when incorporated into an artificial diet [45].
		Cholesterol oxidase expressing tobacco leaves that were incorporated in artificial diets caused mortality and severe stunting of neonate <i>Anthonomus grandis</i> larvae [46].
Lipoxygenases (enzyme)	Dioxygenase enzymes are widely distributed in plants and catalyse the hydroperoxidation of cis-cis-pentadiene moieties in unsaturated fatty acids. Functions by damaging midgut membranes.	Lipoxygenase from soybean retards the growth of <i>Manduca sexta</i> when incorporated into artificial diet [47].
Alpha-amylase inhibitors	Alpha-amylase inhibitors block starch digestion. Widespread in microorganisms, plants and animals, [25,26].	Development of pea weevil larvae (<i>Bruchus pisorum</i> ; Coleoptera) was blocked at an early stage after ingestion of transgenic peas expressing an alpha-amylase inhibitor from the common bean (<i>Phaseolus vulgaris</i>) [48].
Alpha-amylase inhibitors	Alpha-amylase inhibitors block starch digestion. Widespread in microorganisms, plants and animals, [25,26].	Alpha-amylase inhibitor protects against predation by certain species of bruchids (Coleoptera: Bruchidae) and the tomato moth, <i>L. oleracea</i> (Lepidoptera) [49].
		Alpha-amylase inhibitor 1, from the common bean (<i>P. vulgaris</i>), provided complete protection against pea weevil (<i>B. pisorum</i> ; Coleoptera) in transgenic peas. Whereas alpha-amylase inhibitor 2 delayed maturation of larvae [50].
		The alpha-amylase activity in <i>Tecia solanivora</i> larvae was inhibited by alpha-amylase inhibitor from amaranth seeds [51]

Transgene	Source and Mode of Action	Example of use
Protease inhibitors	See section Protease inhibitors for the control of insect pests	See Table 2
Lectins	Multivalent carbohydrate-binding proteins. Some bind to midgut epithelial cells, disrupting their function, causing breakdown of nutrient transport, and absorption of potentially harmful substances [25,52].	<p>Lectin from soybean seed inhibited larval growth of <i>M. sexta</i> [47].</p> <p>Wheatgerm agglutinin was toxic when fed to <i>O. nubilalis</i>. Formation of the peritrophic membrane was disrupted in the anterior midgut microvilli [53].</p> <p><i>O. nubilalis</i> growth was strongly inhibited by wheat germ agglutinin (WGA), whereas <i>M. sexta</i> was not affected. In <i>O. nubilalis</i> larvae, WGA caused hypersecretion of unorganized peritrophic membrane in the anterior midgut lumen, disintegration of microvilli and cessation of feeding [54]</p> <p>The snowdrop lectin (<i>Galanthus nivalis</i>, agglutinin, GNA) reduced <i>L. oleracea</i> larval biomass and slowed larval development when in an artificial diet or expressed in potato plants [55].</p> <p>Transgenic potato expressing snowdrop lectin (<i>G. nivalis</i> agglutinin; GNA) reduced development of <i>L. oleracea</i> larvae. Transgenic plants were significantly less damaged [56].</p> <p>Transgenic tobacco plants expressing leaf (ASAL) and bulb (ASAI) agglutinins from <i>Allium sativum</i> retarded <i>S. littoralis</i> larval development and growth [57].</p> <p>The <i>Moringa oleifera</i> lectin (cMoL) reduced <i>Anagasta kuehniella</i> larval growth and increased development time and pupal mortality when incorporated into an artificial diet [58]</p>
Trypsin-modulating ostatic factor (TMOF)	A peptide that blocks trypsin biosynthesis in mosquitoes (<i>Aedes aegypti</i> ; Diptera [Aea-TMOF]) and fleshflies (Sarcophaga; Diptera) [59].	Injection or oral ingestion of Aea-TMOF caused inhibition of trypsin biosynthesis and larval growth in <i>H. virescens</i> . Mortality of <i>H. virescens</i> increased when fed transgenic tobacco plants expressing Aea-TMOF [60].

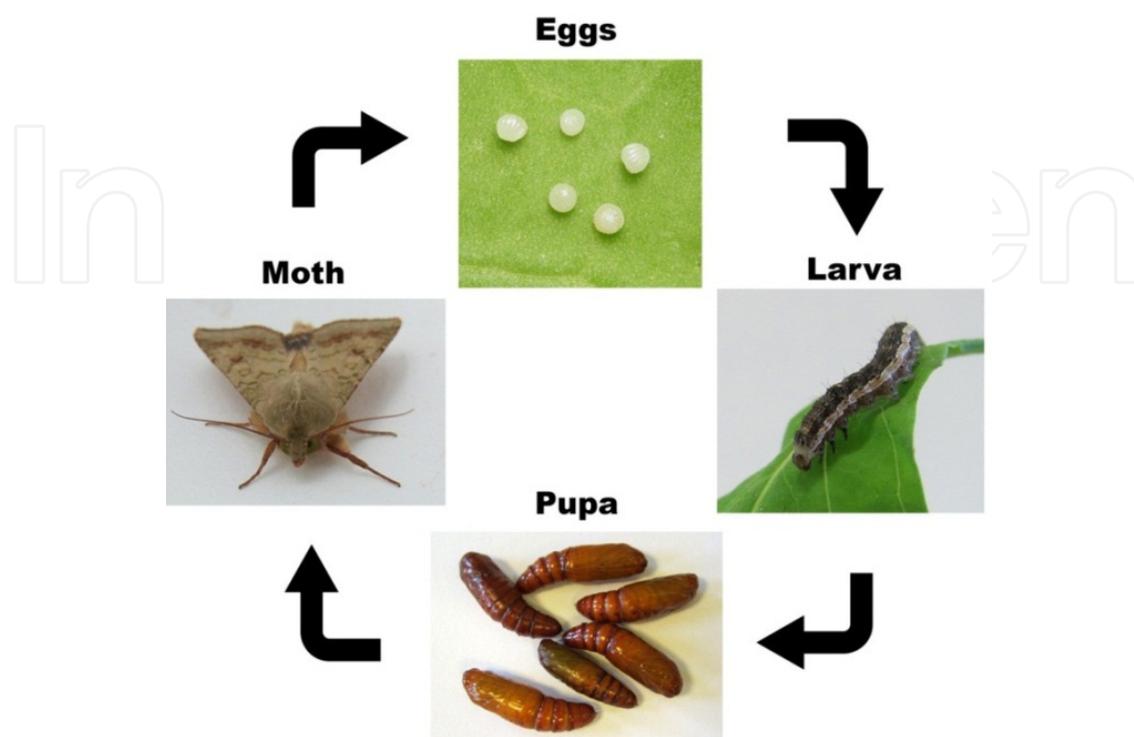
Transgene	Source and Mode of Action	Example of use
Isopentenyl-transferase gene (ipt)	Microorganism-derived gene from <i>Agrobacterium tumefaciens</i> . Codes for a key enzyme in the cytokinin-biosynthetic pathway.	Ipt expressed in tobacco and tomato decreased leaf consumption by <i>M. sexta</i> and reduced survival of the peach potato aphid, <i>Myzus persicae</i> (Hemiptera) [61].
RNAi constructs: 1) Vacuolar ATPase	Nutrient uptake by midgut cells is energized by the electrical difference created by the K ⁺ pump. The K ⁺ pump also regulates midgut lumen pH and determines the potassium concentration in blood, epithelial cells and midgut lumen [62]. The primary motor for transport is a vacuolar-type proton ATPase.	Transgenic corn plants expressing dsRNA of a V-ATPase from <i>Diabrotica virgifera</i> (western corn rootworm [WCR], Coleoptera) showed significant reduction in WCR feeding and plant damage [63].
2) Cytochrome P450 monooxygenase	Cytochrome P450 monooxygenase permits insects to tolerate otherwise inhibitory concentrations of the cotton metabolite, gossypol.	<i>H. armigera</i> fed on plants expressing cytochrome P450 dsRNA had retarded growth. Growth inhibition was more dramatic in the presence of gossypol [64].
3) Hemolin	Recognition of microbial infection is an essential first step in immunity in insects. Induction of this protective effect is associated with up-regulation of microbial pattern recognition protein genes such as hemolin.	Pupae of the giant silkworm (<i>Hyalophora cecropia</i>) were injected with hemolin dsRNA and developed normally into moths. After mating, no larvae emerged from the eggs which had malformed embryos [65]. Prior infection of <i>M. sexta</i> larvae with non-pathogenic <i>E. coli</i> , elicited effective immunity against subsequent infection by the lethal pathogen <i>Photographus luminescens</i> . Injection of hemolin dsRNA left the insect more susceptible to <i>P. luminescens</i> infection than insects that had not experienced prior infection with <i>E. coli</i> [66].

Table 1. Biotechnological approaches for the control of lepidopteran insects with transgenes

1.1. *Helicoverpa* species

Helicoverpa species (Figure 1) are polyphagous pests of at least 181 plant species from 49 families including cotton, corn, soybeans, tobacco and chick-pea [67-69]. They are one of the

most serious pests in cotton-producing countries like Australia, India and China, causing enormous economic problems [70,71].



The lepidopteran species, *H. armigera*, progresses through four stages of development; egg, six larval instars, pupal and adult. The time frame of each of these stages varies with environmental conditions. Over the warmer months, the life cycle can be completed in 30-40 days and each female moth can lay from 500-3000 eggs.

Figure 1. *Helicoverpa armigera* life cycle

One of the reasons these pests are so damaging is the larva's feeding preference for plant structures that are high in nitrogen, principally reproductive structures and growing points such as cotton buds and bolls, corn ears, tobacco buds, and sorghum heads. Damage to these structures has a direct influence on yield [67]. *H. armigera* larvae are foliar feeders at the early instar stage and shift to developing seeds or bolls at later stages [72]. *H. armigera* is a major problem in Australia because it has developed resistance to many of the chemical insecticides that have been used for its control [68,73]. Unlike other lepidopteran species, *H. armigera* larvae don't migrate far from their original host plant, consequently their populations in agricultural areas are exposed to consistent selection pressure, leading to greater resistance to insecticides [5].

In the 1995/96 growing season, transgenic cotton known as Ingard that expressed the *Cry1Ac* gene became commercially available in Australia [71]. To preserve the susceptibility of lepidopterans to Bt toxins, a conservative resistance management plan was imposed, where planting of Ingard cotton was restricted to 30% of the cotton production area per farm [71]. The average amount of insecticide used per hectare was 44% lower on Ingard cotton compared to conventional cotton [71]. In the 2004/05 growing season, Ingard cotton was replaced by Bollgard II, which expressed both the *Cry1Ac* and *Cry2Ab* genes [71].

Restrictions were not placed on this new variety and Bollgard II cotton comprised around 80% of the total cotton area planted in Australia during the 2004/05 and 2005/06 seasons [71] and 95% of the total cotton area in the 2010/2011 season [19]. This reduced the average amount of insecticide used per hectare by 85% compared to conventional cotton [71]. So far, there have been no reported field failures of Bollgard II due to resistance. However, while alleles that confer resistance to Cry1Ac in *H. armigera* are rare in the field, alleles that confer resistance to Cry2Ab are more common.

2. The use of genetically modified plants for control of lepidopteran insects

As mentioned previously, insects are responsible for major crop losses worldwide. In addition to direct impacts on yield, insects also reduce yields by making crops more susceptible to disease causing pathogens [8]. Last decade, most control measures focused on the use of chemical pesticides, a curative pest control strategy that was useful for rapid control of certain pest outbreaks. However, excessive and indiscriminate large-scale use of pesticides has led to development of pesticide-resistant insects [74]. Additionally, the long-term and extensive use of synthetic chemicals has led to concerns regarding their impact on food safety, associated human health and the environment [8]. As the use of pesticides for prevention of insect-associated losses cannot be overlooked in agriculture, there is a greater need to develop alternative or additional technologies which would allow a more selective use of pesticides and provide sustainable crop protection [52]. To achieve this objective, it is necessary to enhance the resistance of plants to pests and pathogens through integrated pest management (IPM) programs. They will need to consist of a combination of control strategies including (A) the use of natural biocontrol factors such as pathogens, predators or parasites [75]; (B) various preventive pest control strategies including crop rotation, intercropping, and cultivation of pest-resistant varieties of plants [8] and (C) genetic control via the release of sterile insects and also the use of natural insecticides. The latter includes secondary metabolites [52,76], viruses [77,78] and transgenes.

As the products of most transgenes are ingested by the insect pest and therefore act through the gut, most of the focus has been on transgene encoded proteins that target the insect midgut and/or the peritrophic membrane to disrupt digestion or nutrition [53,54,79-81]. Generally, the detrimental effects on larval and insect growth result from limited assimilation of nutrients [82-85]. Furthermore, any severe delay in growth and development, in a natural setting, lengthens the period in which the larvae are vulnerable to natural predators such as mice, spiders and predaceous insects [30,86,87]. The use of transgenic plants that express insecticidal agents thus reduces the population of insect pests and reduces the usage of chemical insecticides. This extends the useful life of the insecticides and also reduces the ecological damage they may cause [61]. As with any new method of insect control, the impact of transgenic plants on non-target and beneficial insects, particularly pollinators such as honey bees, needs to be assessed [88-90]. Table 1 lists a number of biotechnology approaches tested on lepidopteran insects. Since the discovery that dsRNA

can silence genes, RNA interference (RNAi) has been developed as an effective tool for regulating gene expression in plants and animals. RNA interference or gene silencing has been used to inhibit virus replication and spread in transgenic plants and has potential to be developed commercially for disease control [91]. The use of RNAi for insect control is less well developed. Insect genes can be down-regulated by injection of dsRNA or by oral administration of high concentrations of exogenously supplied dsRNA as part of an artificial diet, but a much more efficient method of delivering dsRNA is needed before RNAi technology can be used to control pests in the field [64,65]. To date, the most successful transgenes for insect control have been the genes encoding insecticidal toxins from the soil bacterium *Bacillus thuringiensis*.

2.1. The *Bacillus thuringiensis* endotoxins

The use of genes encoding endotoxins from *Bacillus thuringiensis* is now a well-established technology for producing transgenic plants with enhanced resistance to the larvae of lepidopteran insect pests [92]. Bt cotton was first released for commercial production in the USA in 1996 and subsequently grown in several countries including Argentina, Australia, China, Colombia, Indonesia, Mexico, South Africa, and India [93]. Since then other transgenic crop species producing Bt toxins have been commercialized including maize, tomato and potato (<http://cera-gmc.org>). The adoption of Bt crop varieties by farmers has been rapid reflecting the benefits of these crops such as reduced insecticide use, lower production costs and higher yields [94]. Only two Bt crops are grown in Australia (Table 2). In the most recent season (2011/2012) approximately 80% of the cotton grown in Australia was Bollgard II® [95].

B. thuringiensis, a Gram-positive soil bacterium, produces a proteinaceous parasporal crystalline inclusion during sporulation [96]. There are two main categories of Bt toxins: Cry and Cyt. These two groups are classified further by a detailed nomenclature system that describes groups Cry1 to Cry55 and Cyt1 to Cyt2 [97-99]. The Cry toxins are divided into three larger families that are not related phylogenetically. The largest Cry family is the three domain family, and genes from this family are present in the majority of commercialised Bt crops [100].

The larvae of insect orders primarily affected by Bt toxins are Lepidoptera (butterflies and moths), Diptera (mosquitoes) and Coleoptera (larval and adult beetles) [101]. However, Bt toxins are not toxic to people, wildlife, or most beneficial insects [102,103] and therefore the opportunities for biological control are great. The effect of Bt toxins on a range of lepidopteran insects has been studied including: *Bombyx mori* [104], *Helicoverpa armigera* [105], *Heliothis virescens* [106,107], *Manduca sexta* [108,109], *Ostrinia nubilalis* [110-113], *Plutella xylostella* [114,115], *Sesamia nonagrioides* [115], *Spodoptera exigua* [116], *Spodoptera frugiperda* [117] and *Spodoptera littoralis* [118]. The Cry toxins produced in Bt crops generally target lepidopteran pests, although some also target coleopteran pests [100]. The first commercialised Bt crops contained only one Cry toxin, but second generation Bt crops have between two to six different toxins [100].

Trade name	Crop	Bt protein	Company	Year released
Ingard®	cotton	Cry1Ac	Monsanto	1996
Bollgard II®	cotton	Cry1Ac, Cry2Ab	Monsanto	2003

This table lists the transgenic crops in Australia producing Bt proteins.

Table 2. Bt crops grown in Australia

2.2. Mechanism of action

The Bt toxin mechanism of action is described by two models: The pore formation model and the signal transduction model. The initial steps of both models are the same. Upon ingestion by insects the crystalline inclusion is solubilised in the midgut [119]. Most target insects have a high gut pH [120] that is crucial for the efficacy of Bt toxins since most Bt-prototoxins are only soluble above pH 9.5 [121]. The 130 kDa prototoxins are activated by insect gut proteases, which typically cleave from both the C- and N-termini resulting in a 43-65 kDa protease-resistant active core [122-125].

The pore formation model has been the accepted mode of action for 20 years and is supported by numerous publications [96,126-128]. In this model the activated toxins bind to the primary receptors in the brush border membrane of the midgut epithelium columnar cells [14]. The major receptors for Cry toxins in lepidopterans are cadherin-like proteins [129-133]. The binding site of Cry toxins varies depending on the structure of the Cry toxin [105,110]. Binding to cadherin facilitates further proteolytic cleavage of the toxin and promotes the formation of oligomers [128,134]. The toxins then interact with secondary receptors in the midgut larval membrane. These secondary receptors are GPI-anchored proteins; either aminopeptidases or alkaline phosphatases [119,128,131,135]. Following secondary receptor binding, the toxin inserts into the membrane and creates pores [128]. These pores lead to the disruption of membrane integrity and cause an electrolyte imbalance that ultimately leads to death by starvation or septicaemia [136,137]. It is likely that there are more receptors involved in Bt toxicity since insects lacking the cadherin receptor are still killed by modified Bt toxins [138,139].

An alternative model for the Bt toxin mechanism of action proposes that Cry toxins trigger a signalling cascade pathway [140,141]. This model differs from the pore formation model in that it does not involve toxin oligomerisation, secondary receptors or the formation of pores in the membrane. Instead, in this model, binding to the cadherin receptor initiates a Mg²⁺ dependent signal cascade pathway that includes a guanine nucleotide-binding protein, adenylyl cyclase, and protein kinase A which ultimately results in cell death.

2.3. Resistance of lepidopteran insects to Bt toxins

More recently there have been reports of field resistance to Bt crops in pink bollworm (*Pectinophora gossypiella* [142,143]), cotton bollworm (*Helicoverpa* spp [144-147]), armyworm (*Spodoptera frugiperda*[22]) and western corn rootworm (*Diabrotica virgifera virgifera* [148]). Some insects collected from the field have Bt resistance that has been characterized in the

laboratory. However, there is debate about the relevance of this laboratory resistance in the field [149]. A decrease in field performance of Bt corn against *S. frugiperda* was observed in Puerto Rico [150] and against *Busseola fusca* in South Africa [23,151]. In southeastern US problems with control of *H. zea* on Bt cotton have also been reported [144-146].

The most common mechanism of resistance is the disruption of binding of Bt toxin to receptors in the midgut membrane. This disruption may be caused either by mutations in the receptor that blocks binding (reviewed in [20]) or changes in expression of the receptors [152,153]. Mutations in cadherin genes are responsible for Bt resistance in *Heliothis virescens* [154], *Helicoverpa armigera* [155] and *Pectinophora gossypiella* [156]. Another resistance mechanism associated with an ABC transporter locus has been reported in three lepidopteran spp (*H. virescens*, *P. xylostella* and *T. ni* [157]). Resistance to Bt in *Ostrinia nubilalis* is due to reduced midgut protease activity resulting in less activation of the protoxins [111,158,159].

2.4. Management of resistance to Bt crops

There are two main strategies for management of insect resistance to Bt crops: Refuge and pyramiding. The main approach for delaying evolution of resistance to Bt crops is the refuge strategy [21]. Farmers are mandated to maintain an abundance of host non-Bt crops as a refuge surrounding their Bt crops. The theory behind this strategy is that any Bt resistant larvae that arise on the Bt crops will mate with susceptible individuals from neighbouring non-Bt crops. As long as inheritance of resistance remains recessive the offspring will be susceptible to Bt crops [160-162]. This strategy is then combined with several other mandatory farming practices that include control of volunteer and ratoon plants that arise post-harvest, planting within a defined period of time to restrict the exposure of the Bt crop to the insect pests, restricted use of foliar Bt and the cultivation of crop residues [95]. The other major strategy to combat the evolution of Bt resistance is gene pyramiding. For example, the development of second generation Bt cotton that has at least two Bt toxins such as the Monsanto Bollgard II cotton variety, but up to six Bt toxins [100]. Another resistance management strategy which is still in the research phase of development is the use of insecticidal genes with completely different modes of action such as proteinase inhibitors. The success of combining multiple Bt genes for resistance management is contingent on the individual toxins having different targets to prevent cross resistance developing [163-165]. Binding studies with various Cry toxins have been used to identify toxins with different binding sites in the lepidopteran midguts [105,166,167]. This information can be used to design combinations of Cry toxins that complement each other to delay the development of resistance to Bt crops.

In addition to the resistance management plan for Bollgard cotton outlined above, farmers also use integrated pest management (IPM) systems as a sustainable approach to control all pests. IPM systems deploy a tactical combination of biotechnological, chemical, biological and cultural control methods to avoid pest problems [168]. Some of the major IPM strategies and tools include maintenance of beneficial insect populations, ensuring healthy plant growth, managing weed hosts and monitoring pest populations and plant damage regularly. All these

additional practices lead to better control of insect populations in general and therefore helps prevent the development of resistance in insect populations to Bt.

3. Protease inhibitors for the control of insect pests

Protease inhibitors are one component of a plant's natural defence mechanism against herbivores and pathogens [169]. Plants protect themselves directly by constitutively expressing protease inhibitors [170] and by inducing protease inhibitors in response to mechanical wounding or insect attack [169,171]. They may also release volatile compounds after insect damage that function as potent attractants for predators of insect herbivores [172]. The release of volatile compounds after wounding, such as methyl jasmonate also triggers the production of proteinase inhibitors in neighbouring unwounded plants essentially prearming the local population against insect attack [173].

3.1. Mechanism of action of protease inhibitors on lepidopteran insects

Protease inhibitors when incorporated into artificial diets or expressed in transgenic plants increase mortality [174] and reduce the growth and development of larvae from many insect pest species including Coleoptera [175,176], Orthoptera [177] and Lepidoptera [178,179](Table 2). The mechanisms by which ingested PIs mediate their effects on insect physiology differs between insect species [180]. Proteinase inhibitors bind to insect digestive proteases, preventing proteolysis which blocks digestion of protein [181]. This effectively starves the larvae of protein and essential amino acids required for insect growth, development and reproduction [182-185]. To compensate for this inhibition, several insect species increase production of proteases to swamp the ingested PIs [186,187]. This in turn can lead to a limitation in bioavailability of essential amino acids for protein synthesis, impairment of growth and development, and potentially death [182,186]. The loss of the sulphur-containing amino acids (cysteine and methionine) is critical because the sulphhydryl content in trypsin and chymotrypsin is high and procurement of the sulphur-containing amino acids is difficult since cysteine and methionine are in relatively low concentrations in the diet, especially if the food source is plant material [186]. Broadway and colleagues confirmed this hypothesis in bioassays with *Spodoptera exiqua* where the weight-reducing effects obtained with soybean trypsin inhibitor were eliminated when the diets were supplemented with methionine [186].

3.2. PIs in transgenic plants for plant protection: success and failure

Several groups have reported enhanced protection of plants against lepidopteran pests after transformation with genes encoding PIs (Table 3). Despite this substantial body of work, defense strategies based on PI expression in plants have not resulted in any commercial application so far [61,214,215]. This relates to two distinct problems: (1) the levels of PI-expression in transgenic plants and (2) the pest's capacity to react to PI consumption. Most problems arise from the use of a single transgene producing a PI that targets only one protease or one class of protease in the insect midgut.

Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
<i>Arabidopsis thaliana</i> serpin 1 [AtSerpin1]	alpha-1-peptidase inhibitor	Chymotrypsin	<i>Arabidopsis</i>	<i>Spodoptera littoralis</i>	38% biomass reduction after feeding for 4 days [188]
Barley trypsin inhibitor [BTI]	Cereal trypsin inhibitor	Trypsin	Tobacco	<i>Spodoptera exigua</i>	29% reduction in survival [189]
			Wheat	<i>Sitotroga cerealella</i>	No effect on growth or mortality [190]
Bovine pancreatic trypsin inhibitor [BPTI]	Kunitz (animal)	Trypsin, chymotrypsin, plasmin, kallikreins	Tobacco	<i>Spodoptera exigua</i>	Reduced trypsin activity; induced leucine aminopeptidase and carboxypeptidase A activities; chymotrypsin, elastase, and carboxypeptidase B proteases not affected [190]
			Sugarcane	<i>Scirpophaga excerptalis</i>	Significant reduction in weight [191]
Bovine spleen trypsin inhibitor [SI]	Kunitz (animal)	Trypsin, chymotrypsin	Tobacco	<i>Helicoverpa armigera</i>	Reduced survival and growth [192]
Cowpea trypsin inhibitor [CpTI]	Bowman-Birk	Trypsin	Tobacco	<i>Heliothis virescens</i>	Increased mortality [9]
			Tobacco	<i>Helicoverpa zea</i>	Increased mortality [193]
			Rice	<i>Chilo suppressalis-Sesamia inferens</i>	Growth not monitored [11]
			Potato	<i>Lacanobia oleracea</i>	45% biomass reduction [13]
			Tobacco	<i>Spodoptera litura</i>	50% biomass reduction [194]
			Potato	<i>Lacanobia oleracea</i>	Decreased weight and delayed development [12]
Giant taro proteinase inhibitor [GTPI]	Kunitz (plant)	Trypsin, chymotrypsin	Tobacco	<i>Helicoverpa armigera</i>	Decreased growth, no increase in mortality [195]

Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
Mustard trypsin inhibitor 2 [MTI-2]	Brassicaceae proteinase inhibitor	Trypsin, chymotrypsin	Tobacco, <i>Arabidopsis</i> and oilseed rape	<i>Spodoptera littoralis</i>	Increased mortality; surviving larvae up to 39% smaller after 10 days [187]
				<i>Mamestra brassicae</i> , <i>Plutella xylostella</i> , <i>Spodoptera littoralis</i>	<i>P. xylostella</i> : 100% mortality on <i>Arabidopsis</i> ; high mortality & delayed development on oilseed rape. <i>M. brassicae</i> : increased mortality & weight of survivors on <i>Arabidopsis</i> and tobacco, no effect on oilseed rape. <i>S. littoralis</i> : delay in development on oilseed rape [178].
			Tobacco	<i>Spodoptera littoralis</i>	No effect on growth; reduction in fertility [196]
			Oilseed rape	<i>Plutella xylostella</i>	Reduction in survival and weight [30]
<i>Nicotiana glauca</i> protease inhibitor [NaPI]	Proteinase inhibitor II	Trypsin, chymotrypsin	Tobacco	<i>Helicoverpa punctigera</i>	Decreased weight; increased mortality [197]
			Tobacco and peas	<i>Helicoverpa armigera</i>	Increased mortality; delayed growth [198]
			'Royal Gala' apple	<i>Epiphyas postvittana</i>	Larval and pupal weights reduced; developmental abnormalities [31]
			Cotton	<i>Helicoverpa armigera</i>	A higher number of cotton bolls were recorded in plants expressing NaPI and a PotI inhibitor from potato, StPin1A [199].

Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
Potato inhibitor II [Pin II, PPI- II, Pot II, PI-II]	Proteinase inhibitor II	Trypsin, chymotrypsin, oryzin, subtilisin, elastase	Tobacco	<i>Manduca sexta</i>	Growth retarded [200]
			Tobacco	<i>Chrysodeixis eriosoma</i> , <i>Spodoptera litura</i> , <i>Thysanoplusia orichalcea</i>	<i>C. eriosoma</i> larvae grew slower; <i>S. litura</i> and <i>T. orichalcea</i> growth either unaffected or enhanced [201]
			Tobacco	<i>Spodoptera exigua</i>	Growth not affected [202]
			Rice	<i>Sesamia inferens</i>	Decreased weight [74]
			<i>Brassica napus</i>	<i>Plutella xylostella</i>	Lowered growth rates however more plant tissue consumed [203]
Tomato	<i>Heliothis obsoleta</i>	Increased mortality and decreased weight on homozygous plants expressing PI-II and potato carboxypeptidase inhibitor (PCI), opposite effect on hemizygous plants [204]			
<i>Solanum americanum</i> proteinase inhibitor [SaPIN2a]	Proteinase inhibitor II	Trypsin, chymotrypsin	Tobacco	<i>Helicoverpa armigera</i> , <i>Spodoptera litura</i>	Reduction in larval weight and pupation rate [205]
Soybean Kunitz trypsin inhibitor [SBTI, SKTI]	Kunitz (plant)	Trypsin, chymotrypsin, kallikrein, plasmin	Poplar	<i>Clostera anastomosis</i> , <i>Lymantria dispar</i>	Mortality and growth not significantly affected [206]
			Potato	<i>Lacanobia oleracea</i>	Survival and growth decreased by 33% and 40% respectively after 21 days [174]
			Tobacco	<i>Spodoptera litura</i>	Increased mortality and delayed development [207]
			Tobacco	<i>Helicoverpa armigera</i>	Development unaffected [208]

Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
			Tobacco and potato	<i>Spodoptera littoralis</i>	High mortality on tobacco and up to 50% weight reduction on potato [209]
			Sugarcane	<i>Diatraea saccharalis</i>	Increased mortality; retarded growth [210]
Soybean Bowman-Birk trypsin inhibitor [SBBI]	Bowman-Birk	Trypsin, chymotrypsin	Sugarcane	<i>Diatraea saccharalis</i>	Growth severely retarded [210]
			Cauliflower	<i>Plutella xylostella</i> , <i>Spodoptera litura</i>	Increased mortality [42]
			Tobacco	<i>Spodoptera litura</i>	Growth and survival severely retarded [211]
Sweet potato trypsin inhibitor [SWTI, Sporamin]	Kunitz (plant)	Trypsin	Tobacco	<i>Helicoverpa armigera</i>	Increased mortality and delayed growth and development in larvae on plants expressing sporamin and a phytocystatin from taro, CeCPI [212]
			Brassica	<i>Plutella xylostella</i>	Survival rate and body mass was significantly lower in larvae fed plants expressing sporamin and chitinase [213]
Tomato inhibitor I [Tom1]	Proteinase inhibitor I	Chymotrypsin subtilisin, trypsin	Tobacco	<i>Manduca sexta</i>	Little effect on growth [200]
Tomato inhibitor II [TPI-II]	Proteinase inhibitor II	Chymotrypsin trypsin, subtilisin	Tobacco	<i>Manduca sexta</i>	Growth retarded [200]

This table lists plant and non-plant serine protease inhibitors expressed in transgenic plants that have been tested in bioassays with lepidopteran larvae. The major enzymes targeted by each PI are given, however other enzymes may be weakly inhibited or have not been tested.

Table 3. Serine protease inhibitors that have been tested for their effect on growth and development of lepidopteran larvae

The first problem of inadequate levels of PI expression is best exemplified by studies with *P. xylostella*, the diamondback moth. When larvae of the diamondback moth consumed transgenic plants expressing the chymotrypsin and trypsin specific potato type II proteinase inhibitor, Pot II, they suffered lower growth rates. However, this did not confer an advantage to the plants because the larvae consumed more tissue to compensate for their decrease in metabolism [13,203]. As a result, the insects maintained population growth rates similar to those of larvae on non-transgenic plants. Growth enhancement has been reported after PI ingestion in insects from a number of orders [201,216]. Larvae that consumed tobacco leaves expressing low levels of mustard trypsin inhibitor 2 (MTI-2) developed faster, had an increased mean weight and caused more damage to leaves compared to control larvae on non-transgenic tobacco [187]. The increase in leaf surface consumption observed with plants expressing low levels of MTI-2 may have resulted from a decrease in available protein due to the presence of MTI-2 and/or to an increase in gut proteolytic capacity induced by PI consumption [187].

The second problem, the pest's capacity to react to PI consumption, is exemplified by the observation that several PIs that are potent inhibitors of insect proteases *in vitro* fail to produce any deleterious effect when fed to larvae [187]. Several mechanisms have been reported for this lack of effect (Figure 2). For example, the complement of proteolytic enzymes in the insect midgut can be altered after PI ingestion [183,214,217]. This could involve a switch to enzymes of different substrate specificity, but the same mechanistic class. For example, production of a chymotrypsin-like enzyme rather than a trypsin-like protease [195,218]. Another mechanism used to detoxify the PIs is degradation via endogenous proteases within the insect midgut [214,219]. Insects that feed regularly on a particular host plant are generally not affected by the PIs produced by the host. For example the PIs from chickpea, a host plant for *H. armigera*, are rapidly degraded by the *H. armigera* gut proteases [219,220]. Similarly, single domain cystatins from potato multicystatin are degraded when fed to larvae of *Diabrotica* spp (Coleoptera). Sometimes non-host PIs are also rapidly degraded. Human stefin A, a potent inhibitor of human cysteine proteases, was degraded by cystatin-insensitive proteases in the gut of Colorado potato beetle (*Leptinotarsa decemlineata*) and black vine weevil (*Otiorhynchus sulcatus*) [221]. Another anti-PI mechanism is the production of midgut inhibitor-resistant serine proteases [182,222-224]. Some insect larvae adapt to the presence of PIs by replacing the inhibited enzymes with other PI-resistant proteases and can exhibit increased ingestion rates and faster development than larvae fed on control diets lacking PIs [202,204,225,226]. Some classic examples of this phenomenon are as follows. Soybean Kunitz trypsin inhibitor (SKTI) is normally an effective inhibitor of protease activity in gut extracts from *H. armigera* larvae, this insect is not seriously affected by ingestion of this PI because it responds to chronic ingestion of SKTI by increasing activity of an SKTI-resistant trypsin [227]. Similarly, growth and development of *S. exigua* larvae was not impacted when fed leaves from tobacco plants transformed with the chymotrypsin/trypsin specific potato proteinase inhibitor II (Pot II) [202]. Analysis of the trypsin activity in the gut of these insects demonstrated that only 18% of the trypsin activity of insects reared on these

transgenic plants was inhibited by Pot II, whereas 78% of the trypsin activity in the gut of insects reared on control plants was Pot II-inhibitable [202]. The larvae had compensated for the loss of the PI-inhibitable trypsin by a 2.5-fold induction of new activity that was resistant to inhibition by Pot II [202]. Another observation of induction of PI-resistant enzymes was made by Markwick and coworkers who reported that the trypsin in three species of leaf rollers (Tortricidae) that had fed on diets containing SKTI was less inhibited by bovine pancreatic trypsin inhibitor (BPTI) compared to the trypsin in control larvae [228]. These responses have been reported for lepidopteran species that have ingested PIs in native plants, transgenic plants, and artificial diets [195,229]. In summary, potent inhibition of an insect digestive enzyme *in vitro* by a particular PI is not a good prediction that the PI will be useful when expressed as a transgene for crop protection. That is, expression and regulation of midgut serine proteases in herbivorous insects is tightly regulated and is heavily influenced by the levels and the nature of ingested PIs [230,231]. The mechanism by which changes in protease levels and protease isoforms is regulated in response to PI ingestion is still unknown for phytophagous insects. An overview of the effects of PIs on insects is presented in Figure 2.

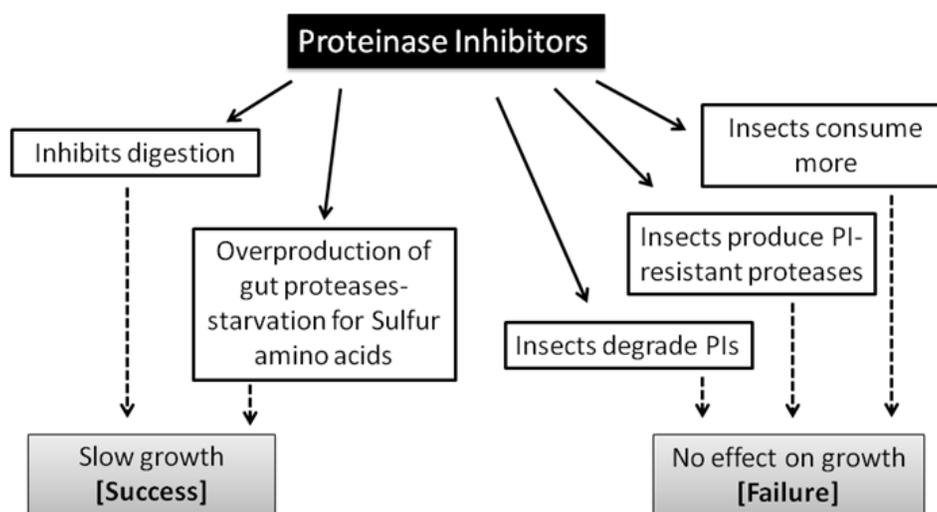
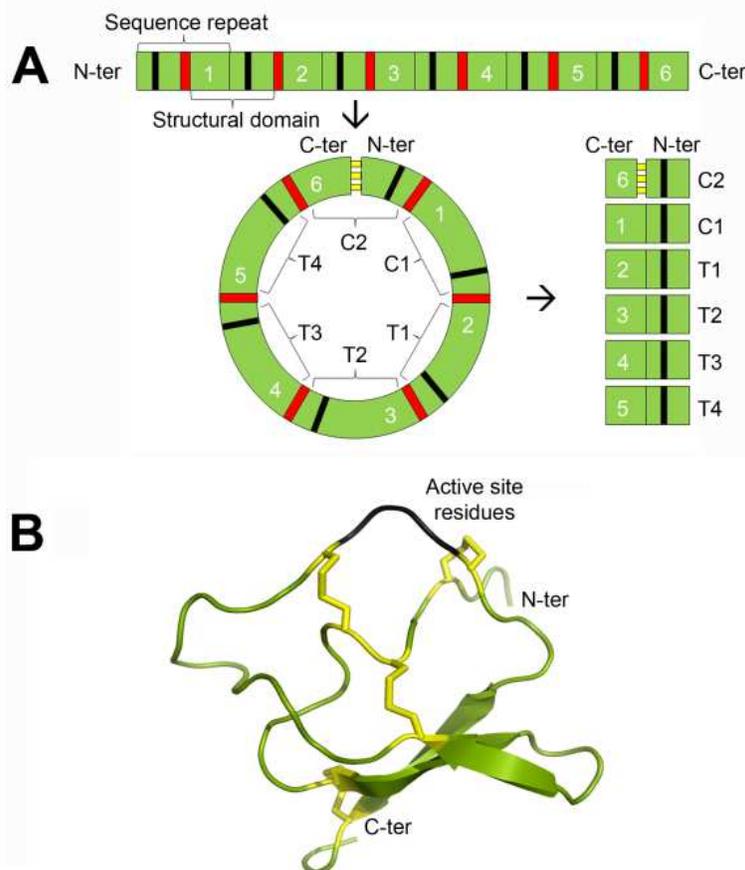


Figure 2. Outline of the various effects of ingested PIs on insect pests leading to success or failure in plant protection

3.3. Proteinase inhibitors from *Nicotiana glauca* as defence molecules against insect pests

Female reproductive tissues and wounded leaves of the ornamental tobacco, *Nicotiana glauca* amass high levels of serine proteinase inhibitors for protection against insect pests and pathogens [232]. These serine proteinase inhibitors (NaPI) belong to the Potato type II family (Merops family I20) which have only been described in the Solanaceae. The NaPI precursor protein (NaProPI; 43 kDa), is composed of an ER signal peptide (29 amino acids), six repeated domains each with a potential PI-reactive site, and a 25 residue C-terminal domain that is essential for vacuolar targeting (VTS) [232-234] (Figure 3).



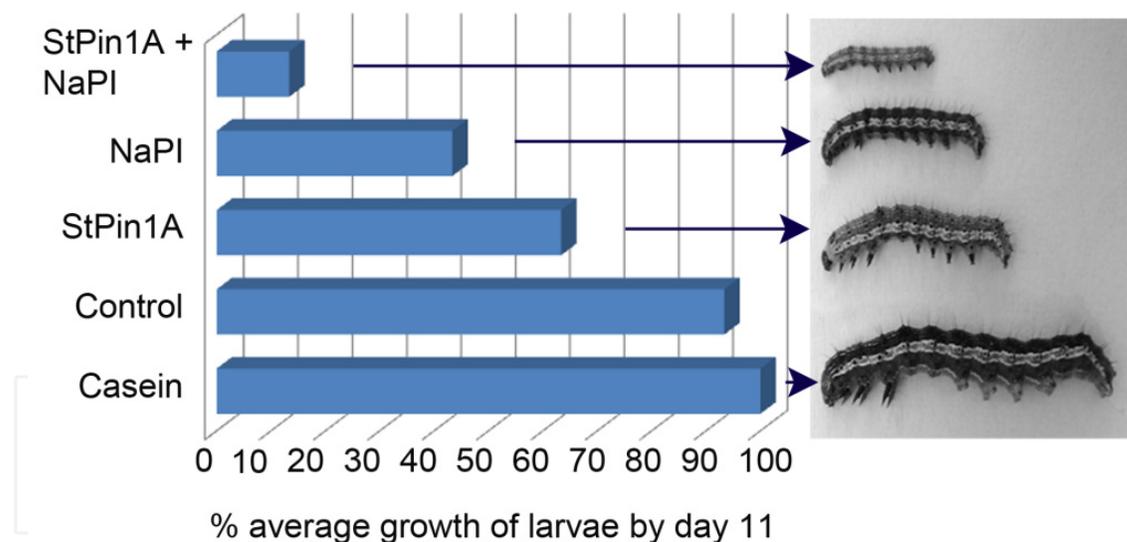
(A) The NaPI precursor protein, shown as a linear gene product, forms a circular 'bracelet' structure that is 'clasped' by three disulphide bonds (yellow) between the N- and C-terminal repeats. Each repeat (labeled 1-6) contains a protease-reactive site (black), which is specific for either chymotrypsin (C1 and C2) or trypsin (T1-4). The six linker regions (red), with sequence EEKKN, are cleaved to release the six active inhibitor domains. The N-terminal signal sequence and the C-terminal vacuolar targeting signal have been omitted for clarity. Figure adapted from Scanlon *et al.* [235]. (B) Ribbon view of T1 showing the major secondary structural element, a triple stranded β -sheet (green) and the cysteines involved in disulfide bonds (yellow). The reactive site residues (black) are positioned between two cysteines that anchor the reactive loop to the central coil [236]. The other five inhibitors have the same structure [236-238].

Figure 3. Diagrammatic representation of the domain organisation of NaProPI and the structure of the T1 inhibitor domain

Processing of NaProPI in the secretory pathway removes the ER signal peptide and VTS, and releases six PIs [232,239]. Processing of the six repeat NaProPI occurs at sites located within, rather than between, these repeated regions [232,239]. Complete removal of the linker sequence (Glu-Glu-Lys-Lys-Asn) contained within each repeated region [239], generates five contiguous inhibitors, a chymotrypsin inhibitor (C1) and four trypsin inhibitors (T1-T4), and two flanking peptides from the N- and C-termini. The flanking peptides form a novel two-chain chymotrypsin inhibitor (C2) that can only be formed if NaPI adopts a circular structure (Figure 3; [240]). The peptides have very similar amino acid sequences [239]. The three-dimensional structures of C1, C2, T1, T2, T3 and T4 have been determined by NMR spectroscopy [234,236,240]. A triple stranded β -sheet is the dominant secondary structural feature; several β -turns and a short region α -helix are also present (Figure 3B; [238]). The reactive site is located on an exposed loop which has a higher degree

of mobility than other regions of the protein (Figure 3B). This is a common feature of PIs and is thought to allow the inhibitor to adapt to slightly different enzymes [239].

Atkinson and colleagues suggested NaPIs may be involved in deterring insects from feeding on stigmas or in protecting the stigma from pathogen invasion since the related type-II PIs from potato and tomato are effective against proteases of fungal, bacterial and insect origin [232,241]. The PIs from *N. alata* inhibit the digestive gut proteases from five insect orders *in vitro* and display significant inhibitory activity against the midgut proteases of *H. punctigera* and *T. commodus* [197,198]. Significant mortality was recorded when *H. punctigera* larvae were fed transgenic tobacco [197] or transgenic peas [198] expressing the NaPI precursor. More recently, the response of *Helicoverpa* larvae to ingestion of NaPI has been more thoroughly characterized. Following ingestion of NaPI, all surviving *Helicoverpa punctigera* larvae produced high levels of a chymotrypsin that was resistant to inhibition by NaPI [199]. However this NaPI-resistant chymotrypsin was strongly inhibited by a potato type 1 inhibitor which is also produced by solanaceous plants, but belongs to a different class of serine proteinase inhibitors. When presented to *H. armigera* larvae in an artificial diet the combination of NaPI and the potato type I inhibitor had a much more dramatic effect on growth and development of the larvae compared to either of the inhibitors alone (Figure 4).



Neonates were transferred to cotton-leaf based artificial diets containing 0.3% of PIs (NaPI, StPin1A) and growth (mg) measured every 2nd day until day 11. Day 11, the % average weights compared to casein control are shown with representative larvae from each treatment (adapted from [199])

Figure 4. Percentage of *Helicoverpa* larval growth on day 11.

This laboratory result was then translated to transgenic plants in the field. Transgenic cotton plants expressing both PI classes, NaPI and StPin1A performed better than transgenic cotton plants expressing either PI alone. The improved performance of the transgenic cotton plants with both PIs was measured by an increase in cotton boll number per plant and increased yield of lint at the end of the cotton growing season (Figure 5)[199].



Figure 5. A higher number of cotton bolls were produced on field grown transgenic cotton producing NaPI and StPin1A (A) compared to Coker (B) the control non-transgenic parent

3.4. Commercialisation of PIs and strategies to avoid resistance

Since the first transgenic plants appeared almost two decades ago, this technology has contributed to the development of new approaches for crop protection [25]. There are numerous reports showing that expression of PIs in transgenic plants confers resistance to the intended target insects (see Table II; reviewed in [61,215,242,243]). However, many of the candidate genes that have been used in genetic transformation of crops have limited application because they do not have broad spectrum activity against the major insect pests or are only mildly effective against the target pests [52]. To overcome the development of insect resistance to transgenic plants expressing PIs, it is necessary to develop PIs that have broad activity against most or all of the proteases that the insects use for digestion. Several strategies have been proposed.

3.4.1. *Selecting second generation protease inhibitors from novel sources*

PI-resistant proteases probably result from the selection pressure imposed on insects when they encounter high endogenous PI levels in certain host plants [170]. Such selection for PI-resistant proteases does not occur for PIs from non-host plants. Therefore, one approach to obtain better inhibitors for a particular insect pest is to search for PIs in plant species that are unrelated to the plant that is the normal host for that pest [10,74,170]. Another approach is to select PIs from synthetic libraries of mutant inhibitors for insect control [170].

3.4.2. *Use of multiple inhibitors*

Another strategy for controlling resistance development is to use at least two inhibitors that have different targets. This can be achieved by producing chimeric proteins, gene stacking (pyramiding) or the use a single inhibitors that have dual targets. Some examples of

bifunctional inhibitors are alpha-amylase/trypsin inhibitors [8] and trypsin/ carboxypeptidase A inhibitors [244]. Similarly, expression of a fusion protein composed of a cystatin and a serine PI has been used to control certain nematode pathogens in transgenic plants [245]. Oppert and colleagues [246] demonstrated synergism between soybean Kunitz trypsin inhibitor and the cysteine protease inhibitor L-trans-epoxysuccinyleucylamide [4-guanidino] butane (E64) in artificial diet bioassays with *Tribolium castaneum* (red flour beetle, Coleoptera).

Transgenic tobacco plants expressing both a Bt-toxin and a cowpea trypsin inhibitor (CpTI) were more protected from *H. armigera* damage compared to transgenic tobacco expressing the Bt-toxin alone [247]. The enhanced insecticidal activity was attributed to enhanced stability of the Bt-toxin when the gut protease activity had been lowered [248,249]. In a separate set of experiments, *H. armigera* and *S. litura* larvae that consumed leaves from transgenic tobacco expressing avidin (from chicken egg white) that had been painted with Cry1Ba protein died significantly faster than larvae given either of the two treatments alone [38]. When used together in bioassays with artificial diet, the different and complementary action of Pot I (a chymotrypsin inhibitor) and CPI (a carboxypeptidase inhibitor) also resulted in a synergistic effect at reducing the growth rate of *Cydia pomonella* (codling moth) larvae [250]. However, the protective effects observed with PI gene constructs have not been sufficient to lead to a serious attempt at commercialising these transgenic crops.

4. Summary

The usefulness of insect-resistant transgenic plants has been widely demonstrated with the highly successfully implementation of crops that produce the Bt toxin. The current fear is that although Bt toxin has defended crops in the field for nearly 10 years now, the discovery of Bt resistance in *H. zea* populations in crop fields in the USA [251] and Bt resistance in populations of *D. virgifera* found in corn fields [148] might lead to widespread development of resistance to the Bt toxin. We have reported that two structurally different PIs that target different enzymes greatly improved the protection of transgenic cotton plants in the field. This supports the general consensus in the literature that no single insect trait will provide sustainable crop protection and that stacking of multiple insect traits that target different mechanisms should be employed.

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