Entomotoxic Plant Proteins: Potential Molecules to Develop Genetically Modified Plants Resistant to Insect-Pests

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Abstract

Insect-pests are detrimental to several crops worldwide and cause significant economic losses in global agriculture. The effective control of insect-pests in agriculture demands different strategies, which vary from preventive cultural practices, mechanical control, chemical control, biological control, and the use of resistant plant varieties. When there is no natural plant genotype genetically resistant to insect-pests, development of genetically modified (GM) resistant plants is an option. The expression of bacterial *Bacillus thuringiensis* (Bt) entomotoxins in GM plants has been successfully applied in field conditions over the past few decades. Nevertheless, there are alternative entomotoxic proteins from plant sources, which may be synergistically used in the GM plant Bt strategy for the control of insect-pests. This review presents the biochemical properties and mechanisms of action of the most commonly described plant protein entomotoxins, including lectins, enzymes (ribosome-inactivating proteins (RIPs), ureases and urease-derived encrypted peptides, chitinases and proteases/peptidases/proteinases), inhibitors of insect digestive enzymes (protease inhibitors and α-amylase inhibitors), and peptides (defensins and cyclotides). In addition, this review discusses the potential application of plant entomotoxic proteins to develop durable control of insect-pests via GM plant strategies.

Keywords

Transgenic plant • Lectins • Plant enzymes • Inhibitors of insect digestive enzymes • Insecticidal peptides

Introduction

Insect-pests cause significant economic losses in global agriculture and are detrimental to several crops worldwide. Although plants lack an immune system that is comparable to animals, plants have evolved an array of structural and chemical defense mechanisms to counteract insect attacks (the reader is referred to

► Chap. 1, "General Mechanisms of Plant Defense and Plant Toxins"). In any case, plant defense mechanisms against insect-pests may be either constitutive or induced. Constitutive defenses are continuous and include physical barriers, such as thick cell walls and waxy epidermal cuticles. In addition to these preformed barriers, plant cells respond to insect attacks with inducible chemical defenses that include (a) the production of substances that attract natural enemies to insect-pests, (b) the production of entomotoxic molecules that directly act upon the insect-pest survival rate, or (c) the production of molecules involved in plant programmed cell death (apoptosis), all of which oppose insect damage. The biochemical nature of entomotoxins may be secondary metabolites, microRNAs, and proteins (Van Loon et al. 2006; Barbehenn and Constabel 2011; Birkett and Pickett 2014; Younis et al. 2014). Plants express these entomotoxins in various tissues. The highest expression is typically observed in storage organs, such as seeds and tubers, particularly upon wounding or attack by pests (Dang and Van Damme 2015).

Here, the biochemical properties and mechanisms of action of the most commonly described plant entomotoxic proteins, including lectins, enzymes (ribosome-inactivating proteins-RIPs, ureases and urease-derived encrypted peptides, chitinases, and proteases), inhibitors of insect digestive enzymes (protease inhibitors and α-amylase inhibitors), and peptides (defensins and cyclotides) are presented. These entomotoxin categories are provided for instructive purposes, with the understanding that various toxins may fall into more than one category. For example, entomotoxic defensins are both peptides and α-amylase inhibitors; RIPs are both lectins and enzymes; and ureases, although they are enzymes, do not fully exert their insecticidal action through enzymatic processes. Entomotoxic plant proteins also fall into various pathogenesis-related protein (PR protein) categories that are induced upon pest attack (Van Loon et al. 2006). PR proteins are divided into families denoted PR-1 to PR-17 (Van Loon et al. 2006). Accordingly, the present review addresses the PR-3, PR-4, PR-8, and PR-11 families that comprise the chitinases; the PR-6 family, which includes the protease inhibitors; the PR-10 family that includes the ribonucleases, such as RIPs; and the PR-12 family, which comprises the defensins.

The effective control of insect-pests in agriculture demands various strategies, which vary from preventive cultural practices, mechanical, chemical (synthetic pesticides), or biological control (entomopathogenic microorganisms and insect natural enemies) and the use of resistant plant varieties. When there is no natural source of plant genetically resistant to insect-pests, development of genetically modified (GM) resistant plants is an option. The expression of bacterial *Bacillus thuringiensis* (Bt) entomotoxins in GM plants has been successfully applied in field conditions over the past few decades (Lucena et al. 2014; Palma et al. 2014). Nevertheless, Bt entomotoxins have some limitations, such as the low toxicity against sap-sucking insects (Chougule and Bonning 2012). Fortunately, there is a wide range of alternative entomotoxic proteins from plant sources that may be used in GM plant strategies in synergy with the Bt technology to control insect-pests. Therefore, the present report focuses on the description of several insecticidal proteins isolated from plant sources that can be used in a GM plant approach to control insect-pests.

Lectins: One of the First Recognized Classes of Plant Molecules with Insecticidal Properties

Lectins are carbohydrate-binding proteins produced by algae, plants, and animals and belong to the innate immune system, among other physiological functions (Macedo et al. 2015a). Various lectins were reported from different plant species and were found at high concentrations in many tissues, such as seeds, bulbs, and barks (Macedo et al. 2015a). It was shown that some of these lectins were able to agglutinate erythrocytes of a specific human blood group within the ABO system. This discovery was the reason for the name "lectin," which comes from the Latin verb "legere," which means "to select." Therefore, other names for lectins are also applied, such as agglutinins and hemagglutinins, although the first is the most commonly used.

Plant lectins can be broadly classified into four groups, based on the number of domains: (i) merolectins have a single carbohydrate-binding domain and do not possess agglutinating activity, (ii) hololectins contain multiple carbohydrate-binding sites, (iii) chimerolectins possess a carbohydrate-binding domain and an additional domain conferring other biological activities, and (iv) superlectins have multiple carbohydrate domains that recognize structurally unrelated sugars (Macedo et al. 2015a).

Plant lectins can bind to the monosaccharides and oligosaccharides present in animal, fungal, and insect cells. Several different carbohydrate-binding domains have been identified in plant lectins that interact with insect-pest glycans (Macedo et al. 2015a). Hence, plant lectins evolved the ability to negatively interact and interfere with the growth and physiological functions of different insect species, resulting in their entomotoxic properties (Macedo et al. 2015a). The insecticidal activity of plant lectins against a wide range of Coleoptera, Homoptera, Diptera, and Lepidoptera insect species is well documented in the literature. Therefore, plant lectins represent a potential naturally occurring insecticide tool that can be applied to protect crops against insect-pests.

Acetylglucosamine-Binding Lectins

Some plant lectins bind specifically to the carbohydrate molecule *N*-acetyl-D-glucosamine (GlcNAc) that is the monomer of chitin, present in fungal cell walls, nematode egg shells, insect and crustacean exoskeletons, and insect peritrophic membranes, but is not produced by plants.

There are numerous reports of plant GlcNAc-binding lectins with demonstrated entomotoxic activity. For instance, the GlcNAc-binding wheat germ lectin WGA was able to inhibit the growth of the cowpea seed beetle (*Callosobruchus maculatus*), the Southern corn rootworm (*Diabrotica undecimpunctata*), and the European corn borer (*Ostrinia nubilalis*), when tested in artificial diets (Murdock et al. 1990; Czapla and Lang 1990). Although WGA was active against coleopteran

and lepidopteran insects, it did not exhibit an effect against hemipteran species (Vandenborre et al. 2011).

Mannose-Binding Lectins

Certain lectins exhibit specificity to α-D-mannose molecules, such as the snowdrop lectin, also denoted GNA (*Galanthus nivalis* agglutinin). Reports demonstrated GNA activity against important plant pests, such as the rice brown planthopper (*Nilaparvata lugens*) and bruchid beetles (Powell et al. 1993; Gatehouse et al. 1998), but there was no effect on mammals (Pusztai 1991). The ingestion of the lectin GNA by insect-pests induces modifications of the insect gut brush border marker enzymes (Pusztai 1991). GNA was also the first plant insecticidal lectin to be transformed into a plant and tested against specific insect-pests. GNA expression in GM potato plants protected against damage by the tomato moth *Lacanobia oleracea* (Table 1). Interestingly, the GNA expressed by GM potato plants did not affect the nontarget ectoparasitoid wasp *Eulophus pennicornis* (Bell et al. 2001). Moreover, an analysis of the tritrophic interaction between the GM potato expressing GNA, the peach potato aphid (*Myzus persicae*), and the beneficial predator 2-spot ladybird (*Adalia bipunctata*) suggested that GNA is not a deterrent to the nontarget ladybird insects (Down et al. 2003). GNA was also evaluated in GM rice (*Oryza sativa*) plants under the control of a phloem promoter. Bioassays with GM rice expressing GNA in the phloem tissue demonstrated that the lectin reduced insect fecundity and survival, inhibited insect development, and altered the feeding pattern of *N. lugens* (Table 1).

There are reports of lectins being used in fusion proteins with other entomotoxins as an alternative to facilitate the delivery of the fused insecticidal protein. For instance, GNA was used as a carrier of the spider venom neurotoxin from *Segestria florentina,* denoted SFI1 (Fitches et al. 2004). In this case, the GNA-SFI1 fusion protein was expressed in *Pichia pastoris*, and the purified recombinant fusion protein was evaluated against the larvae of *L. oleracea*. It was observed that GNA could carry SFI1 through the hemolymph of lepidopteran larvae, increasing the toxic effects of the SF1 venom (Fitches et al. 2004). The GNA-SF1 fusion protein was also tested in vitro against *N. lugens* and *M. persicae* (Down et al. 2006). Although the best results were observed against *N. lugens*, the GNA-SFI1 fusion protein was also toxic to *M. persicae* (Down et al. 2006).

Concanavalin A (ConA), a mannose-glucose lectin isolated from jack bean (*Canavalia ensiformis*), exhibits high activity against hemipteran insects, including the pea aphid *Acyrthosiphon pisum* (Sauvion et al. 2004a). ConA exerted deleterious effects upon the epithelial cells of the insect gut, leading to hypersecretion and a progressive detachment of the apical membrane (Sauvion et al. 2004b). Therefore, it was suggested that ConA binds to the glycosylated receptors on the surface of the insect gut cells, affecting their metabolism and function.

The gene encoding the mannose-binding lectin ZGA, which was isolated from the Chinese medicinal herb *Zephyranthes grandiflora*, was introduced into tobacco

Table 1 Entomotoxic plant lectins expressed in GM plants

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[b]
Allium sativum	ASAL	*Myzus persicae*	*Nicotiana tabacum*	Dutta et al. 2005
		Nephotettix virescens; Nilaparvata lugens	*Oryza sativa*	Saha et al. 2006; Chandrasekhar et al. 2014
		Aphis craccivora	*Cicer arietinum*	Chakraborti et al. 2009
		Myzus nicotianae; Spodoptera littoralis	*Nicotiana tabacum*	Sadeghi et al. 2007; Sadeghi et al. 2008
	ASA II	*Myzus nicotianae; Spodoptera littoralis*	*Nicotiana tabacum*	Sadeghi et al. 2007; Sadeghi et al. 2008
Allium sativum and *Galanthus nivalis*	ASAL + GNA[a]	*Nilaparvata lugens; Nephotettix virescens; Sogatella furcifera*	*Oryza sativa*	Bharathi et al. 2011
Amaranthus caudatus	ACA	*Aphis gossypii*	*Gossypium tabacum*	Wu et al. 2006
Canavalia ensiformis	ConA	*Lacanobia oleracea; Myzus persicae*	*Solanum tuberosum*	Gatehouse et al. 1999
Galanthus nivalis	GNA	*Aulacorthum solani; Lacanobia oleracea; Myzus persicae; Nephotettix cincticeps; Nilaparvata lugens*	*Solanum tuberosum*	Down et al. 1996; Powell et al. 1993; Gatehouse et al. 1997; Fitches et al. 1997; Down et al. 2003
		Cnaphalocrocis medinalis; Laodelphax striatellus; Nephotettix virescens; Nilaparvata lugens; Scirpophaga incertulas	*Oryza sativa*	Rao et al. 1998; Foissac et al. 2000; Maqbool et al. 2001; Sun et al. 2002

(*continued*)

Table 1 (continued)

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[b]
		Diatraea saccharalis; Eoreuma loftini	Saccharum *officinarum*	Setamou et al. 2002
		Helicoverpa zea; Myzus persicae	*Nicotiana tabacum*	Hilder et al. 1995; Wang and Guo 1999
		Sitobion avenae	*Triticum aestivum*	Stoger et al. 1999
Glycine max	SBL	*Spodoptera exigua*	*Nicotiana tabacum*	Guo et al. 2013
Helianthus tuberosus	HTA	*Myzus persicae*	*Nicotiana tabacum*	Chang et al. 2003
Oryza sativa	Orysata	*Acyrthosiphon pisum; Myzus persicae; Spodoptera exigua*	*Nicotiana tabacum*	Al Atalah et al. 2014
Phaseolus vulgaris	PHA	*Lacanobia oleracea*	*Arabidopsis thaliana*	Fitches et al. 2001
Pisum sativum	PSA	*Heliothis virescens*	*Nicotiana tabacum*	Boulter et al. 1990
Pinellia ternata	Pta + Cry1Ac[a]	*Myzus persicae; Plutella xylostella*	*Isatis indigotica*	Xiao et al. 2012
Triticum aestivum	WGA	*Diabrotica undecimpunctata; Ostrinia nubilalis*	*Zea mays*	Maddock et al. 1991
		Lipaphis erysimi	*Brassica juncea*	Kanrar et al. 2002
Zephyranthes grandiflora	ZGA	*Myzus nicotianae*	*Nicotiana tabacum*	Ye et al. 2009

[a]Pyramided genes within the same GM plant line

[b]**Al Atalah et al**. 2014, Plant Sci, 221–222:21–28; **Bharathi et al**. 2011, J Biotechnol 152 (3):63–71; **Chakraborti et al**. 2009, Transgenic Res 18(4):529–544; **Chandrasekhar et al**. 2014, Biotechnol Lett 36(5):1059–1067; **Chang et al**. 2003, Transgenic Res 12:607–614; **Down et al**. 1996, J Insect Physiol 42(11):1035–1045; **Down et al**. 2003, Transgenic Res 12(2):229–241; **Dutta et al**. 2005, Plant Biotechnol J 3:601–611; **Fitches et al**. 1997, J Insect Physiol 43 (8):727–739; **Fitches et al**. 2001, J Insect Physiol 47(12):1389–1398; **Foissac et al**. 2000, J Insect Physiol 46(4):573–583; **Gatehouse et al**. 1997, Mol Breed 3(1):49–63; **Gatehouse et al**. 1999, Mol Breed 5(2):153–165; **Guo et al**. 2013, Plant Sci 211:17–22; **Hilder et al**. 1995, Transgenic Res 4(1):18–25; **Kanrar et al**. 2002, Plant Cell Rep 20:976–981; **Maddock et al**. 1991, Third Int Congress Plant Mol Biol, Tucson, Arizona-USA; **Maqbool et al**. 2001, Mol Breed 7:85–93; **Powell et al**. 1993, Entomol Exp Appl 66(2):119–126; **Rao et al**. 1998, Plant J 15(4):469–477; **Sadeghi et al**. 2007, Pest Manag Sci 63:1215–1223; **Sadeghi et al**. 2008, Transgenic Res 7:9–18; **Saha et al**. 2006, Planta 223:1329–1343; **Setamou et al**. 2002, J Econ Entomol 95(2):469–477; **Stoger et al**. 1999, Mol Breed 5(1):65–73; **Sun et al**. 2002, Crop Prot 21(6):511–514; **Wang et al**. 1999, Chin Sci Bull 44(22):2051–2058; **Wu et al**. 2006, Plant Breed 125:390–394; **Xiao et al**. 2012, Mol Biol Rep 39(1):485–491; **Ye et al**. 2009, Appl Biochem Biotechnol 158:615–630

(*Nicotiana tabacum*) plants and tested against the tobacco aphid *Myzus nicotianae* (Table 1). An *in planta* bioassay with GM plants expressing ZGA showed a significant effect on aphid survival and fecundity (Table 1).

Tobacco plants transformed with mannose-binding lectin ASAL from garlic (*Allium sativum*) leaves displayed insecticide activity towards *M. persicae* (Table 1). The physicochemical features of the recombinant ASAL were the same as the native protein, indicating that the development of GM plants expressing ASAL could be an alternative tool for insect-pest control (Table 1). The lectin ASAL was later introduced into rice, and the resulting GM plants were evaluated in bioassays against the sap-sucking insect-pests *lugens* and *Nephotettix virescens (green leafhopper).* ASAL caused an approximately 40 % increase in insect mortality and a 30 % reduction of insect fecundity (Table 1). ASAL was also used to transform chickpea (*Cicer arietinum*) plants, and the resulting GM plants were challenged with the phloem-feeding cowpea/groundnut aphid *Aphis craccivora* (Table 1). The ASAL expressed by the GM chickpea caused an 18.5 % reduction in insect survival and a 32 % reduction in insect fecundity (Table 1). When transgenically expressed in tobacco (*N. tabacum*) plants, both the garlic leaf ASAL and the garlic bulb ASAII lectin conferred resistance to *M. nicotianae* (Table 1). Similar experiments showed that when ASAL or ASAII was expressed in GM tobacco, the weights of *Spodoptera littoralis* (cotton leafworm) larvae were significantly decreased, which caused a delay in their development and metamorphosis (Table 1), confirming the potential of the ASAL lectin for insect-pest control. Recently, it was demonstrated that ASAL expression under a phloem-specific promoter in GM rice resulted in resistance to the sap-sucking hopper *N. lugens* (Table 1). Insect bioassays on T2 homozygous rice lines expressing ASAL in the phloem tissue revealed an approximately 80 % reduction in the survival, development, and fecundity of *N. lugens* compared to the wild-type plants (Table 1). Interestingly, ASAL does not possess any apparent features of an allergen, which indicates that it is biosafe for food purposes (Mondal et al. 2011).

Pyramided GM rice lines expressing the garlic lectin ASAL and the snowdrop lectin GNA were developed through sexual crosses between two stable GM rice lines containing either of the lectin genes (Table 1). When challenged with three major sap-sucking pests of rice, *N. lugens*, *N. virescens*, and white-backed planthopper (*Sogatella furcifera*), the resulting homozygous F3 pyramided GM rice plants displayed an enhanced capability to reduce insect survival, fecundity, and feeding ability, in addition to delaying the development of the pest compared to the parental GM lines (Table 1).

Entomotoxic Lectins Expressed in GM Plants

Various lectins have been used in experiments to protect plants against insect-pests via GM plant strategies, as mentioned above and below and summarized in Table 1. The ACA lectin from *Amaranthus caudatus*, whose carbohydrate-binding nature was not studied, provided the plant an increased resistance toward the melon and

cotton aphid *Aphis gossypii* when introduced into cotton (*Gossypium* sp.) plants and expressed directly in the phloem tissue (Table 1). Additionally, the HTA lectin from the Jerusalem artichoke (*Helianthus tuberosus*), whose carbohydrate-binding target is unknown, reduced the development and fecundity of *M. persicae* when expressed in GM tobacco plants (Table 1). Some other examples of GM plants expressing plant lectins and displaying resistance against herbivores are presented in Table 1.

To date, there are no commercially available varieties of lectin-expressing GM crops. Prior to market availability, it is essential to perform a biosafety assessment of GM lectin-expressing plants. The toxicity of insecticidal plant lectins in mammals was investigated, and in rare cases, adverse effects can be observed (Macedo et al. 2015a), implicating that the food biosafety of plant lectin-based GM crops should be monitored on a case-by-case basis. It is equally necessary to analyze the environmental biosafety of GM crops expressing plant lectins towards nontarget organisms, such as the insect-pests' natural enemies and beneficial fungi and insects, which frequently possess carbohydrates recognized by specific lectins.

Alternatively, the deleterious effects of some plant lectins upon mammal cells may be used to develop drugs against cancer. It is known that some plant lectins affect both apoptosis and autophagy by modulating the signaling pathways that are specifically involved in cancer (Jiang et al. 2015). Therefore, plant lectins have great potential for the development of novel antitumoral agents (the reader is referred to ▶ Chap. 18, "Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities").

Moreover, the use of lectins in protecting GM plants against insect-pests may be interesting if lectins are used as carriers of other entomotoxins. Several lectins exhibit a strong resistance to insect gut proteolysis, which favors the lectin-carbohydrate interaction and, consequently, the lectin's toxicity. This feature of lectins is being explored for the delivery of other insecticidal proteins to the optimal sites within the target insect by creating fusion proteins with lectins (Macedo et al. 2015a). When ingested orally by the insect, the potency of some entomotoxins is low because they do not effectively reach the hemolymph to exert their insecticidal activity. Hence, the entomotoxin fusion with lectin as a carrier endows the fused protein with the ability to cross the target insect's gut epithelium and reach the hemolymph without being degraded (Macedo et al. 2015a). These observations demonstrate the promising use of plant lectins as entomotoxin carriers for the control of insect-pests.

Plant Enzymes as Weapons Against Insect-Pests

Ribosome-Inactivating Proteins (RIPs)

Ribosome-inactivating proteins (RIPs) include a group of toxins that are widely distributed in the plant kingdom, as well as in some fungi, algae, and bacteria, and consist of protein synthesis inhibitors that operate at the ribosomal level (Virgilio et al. 2010; Stirpe 2013; the reader is also referred to ▶ Chaps. 16,

"Biotechnological Potential of Ribosome-Inactivating Proteins (RIPs)," ▶ 17, "Toxic but Exploitable Actions of Ribosome-Inactivating Proteins," ▶ 7, "Ribosome-Inactivating Proteins: An Overview," and ▶ 8, "Plant AB Toxins with Lectin Domains." RIPs exhibit an RNA *N*-glycosidase activity that specifically depurinates an adenine base from large ribosomal RNA molecules (Virgilio et al. 2010; Stirpe 2013). Interestingly, there are reports of certain RIPs that exhibit DNase, superoxide dismutase, or phospholipase activity (Virgilio et al. 2010) in addition to the typical RNA glycosidase activity.

Based on the molecular structure, there are two groups of RIPs: type I RIPs, which are composed of a single peptide chain, and type II RIPs, which are heterodimeric proteins composed of two peptide chains, i.e., A and B chains. The A chain exhibits *N*-glucosidase activity on the ribosomal RNA, whereas the B chain contains a carbohydrate-binding domain, also known as a lectin domain (Virgilio et al. 2010; Stirpe 2013).

RIPs have a natural role in plant resistance against several insect-pests (Virgilio et al. 2010). Usually, plant RIPs specifically recognize the galactosyl termini of glycoproteins present on the cell surface of insect-pests, which facilitates the uptake of RIPs through the endocytic pathway. After reaching the cytoplasm, RIPs exert their enzymatic activity on the ribosomal RNA, resulting in target cell death by apoptosis.

Castor bean (*Ricinus communis*) ricin, a classic and well-studied seed type II RIP, contains an A chain (30 kDa) that cleaves the *N*-glycosidic bond of an adenine residue from an exposed loop of the eukaryotic 28S ribosomal RNA, thereby interrupting protein synthesis and leading to cell death (Virgilio et al. 2010; Stirpe 2013). Ricin is highly toxic against a variety of insects, although the level of activity varies according to the insect order (Carlini and Grossi-de-Sá 2002).

Type I RIPs have a similar sequence and mode of action as that of the ricin A chain (Carlini and Grossi-de-Sá 2002). There are various insecticidal type I RIPs in plants that have been characterized in the literature, including the pokeweed antiviral protein (PAP) (from *Phytolacca americana*), lychnis (from *Lychnis chalcedonica*), momordin (from *Momordica charantia*), and gelonin (from *Gelonium multiflorum*), all of which are active against *Anticarsia gemmatalis* (velvetbean moth/caterpillar) and *Spodoptera frugiperda* (fall armyworm); saporin (from *Saponaria officinalis*), which is toxic to *C. maculatus*, *A. gemmatalis,* and *S. frugiperda*; and numerous other entomotoxic type I RIPs (Carlini and Grossi-de-Sá 2002).

Nevertheless, there are few reports on GM plants expressing plant RIPs that are resistant to insect-pests, as indicated in Table 2. *N. tabacum* lines expressing an activated form of a maize RIP, denoted MRIP, showed resistance towards the larvae of the cigarette beetle (*Lasioderma serricorne*), the tobacco hornworm (*Manduca sexta*), and the corn earworm (*Helicoverpa zea*) (Table 2). Additionally, crossings of the abovementioned MRIP expressing tobacco plants with a line expressing a plant peroxidase resulted in a GM *N. tabacum* resistant to H. zea and L. serricorne (Table 2). GM maize (*Zea mays*) plants expressing both MRIP and the wheat germ

Table 2 Entomotoxic plant ribosome-inactivating proteins (RIPs) expressed in GM plants

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[b]
Sambucus nigra	SNA-I	*Myzus nicotianae; Spodoptera exigua*	*Nicotiana tabacum*	Shahidi-Noghabi et al. 2009
Zea mays	MRIP	*Helicoverpa zea; Lasioderma serricorne; Manduca sexta*	*Nicotiana tabacum*	Dowd et al. 2003
	MRIP + Tobacco Peroxidase[a]	*Helicoverpa zea; Lasioderma serricorne*	*Nicotiana tabacum*	Dowd et al. 2006
	MRIP + Wheat WGA Lectin[a]	*Helicoverpa zea; Spodoptera frugiperda*	*Zea mays*	Dowd et al. 2012

[a]Pyramided genes within the same GM plant line
[b]**Dowd et al**. 2003, J Agric Food Chem 51:3568–3574; **Dowd et al**. 2006, J Agric Food Chem 54:2629–2634; **Dowd et al**. 2012, J Agric Food Chem 60:10768–10775; **Shahidi-Noghabi et al**. 2009, Transgenic Res 18:249–259

lectin WGA were resistant to feeding by *S. frugiperda* and *H. zea* larvae (Table 2). Furthermore, GM *N. tabacum* expressing SNA-I (*Sambucus nigra* agglutinin-I) was resistant to *M. nicotianae* and the beet armyworm (*Spodoptera exigua*) (Table 2).

In addition to their usual entomotoxicity, RIPs sometimes can be toxic to mammals and other nontarget organisms (Virgilio et al. 2010). Nevertheless, the antitumoral and antiviral (Virgilio et al. 2010; Kaur et al. 2011; Stirpe 2013) properties of several plant RIPs are promising for drug development (the reader is referred to ▶ Chaps. 18, "Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities," and ▶ 4, "Plant Toxins as Sources of Drugs.")

Ureases and Urease-Derived Encrypted Peptides

Ureases are metalloenzymes that hydrolyze urea into ammonia and carbon dioxide and are found in plants, fungi, and bacteria (Stanisçuaski and Carlini 2012; the reader is also referred to ▶ Chap. 9, "Moonlighting Toxins: Ureases and Beyond". The urease from jack bean seeds was the first enzyme to be crystallized and consists of a homohexamer of individual 90.7 kDa chains (Sumner 1926). The main role of plant ureases is to allow the use of external and internal urea as a nitrogen source. Because ureases are abundant within the seeds of several plant species, seed ureases putatively promote embryo germination through the hydrolysis of the stored nitrogen sources (Stanisçuaski and Carlini 2012). Additionally, plant ureases exhibit insecticidal and antifungal activities. Therefore, seed ureases also play a major role

in the protection of the embryo against pathogenic fungi and insect-pests during germination (Stanisçuaski and Carlini 2012). The insecticidal activity of ureases is completely independent from their enzymatic activity and involves the release of urease-derived peptides after hydrolysis by the insect's digestive enzymes (Stanisçuaski and Carlini 2012). Hence, the entomotoxic peptides derived from urease hydrolysis inside insect midguts are referred to as urease-derived encrypted peptides in this chapter.

Interestingly, insects such as *C. maculatus* and *Rhodnius prolixus* (kissing bug) that produce cathepsin-like enzymes (cysteine and aspartic proteases) in their digestive tract are susceptible to urease, whereas insects that have trypsin-like digestive enzymes (serine proteases), such as *M. sexta*, *Schistocerca americana* (locust), *Drosophila melanogaster* (fruit fly), and *Aedes aegypti* (yellow fever mosquito), are not susceptible to ureases (Stanisçuaski and Carlini 2012). The differential processing of ureases by the insect's digestive enzymes in different stages of the insect life cycle affects the distinct susceptibility of adult and nymph pests, and mortality is correlated with the release of the entomotoxic peptides (Stanisçuaski and Carlini 2012).

The insecticidal activity of the major jackbean urease isoform JBURE-I (approximately 90 kDa each monomer) primarily depends on the release of the entomotoxic urease-derived encrypted peptide pepcanatox (approximately 10 kDa) by insect gut cathepsin-like enzymes (Ferreira-da-Silva et al. 2000). Based on the sequence of pepcanatox, a recombinant peptide named Jaburetox was produced (Mulinari et al. 2007). The recombinant Jaburetox urease-derived encrypted peptide, with approximately 11 kDa, is toxic to various insect-pests, including species that are not affected by the native urease JBURE-I (Stanisçuaski and Carlini 2012). Jaburetox modeling and computational simulations identified structural motifs similar to those found in pore-forming proteins (Mulinari et al. 2007), suggesting that Jaburetox anchors in polar-nonpolar interfaces (Barros et al. 2009). Moreover, it was demonstrated that Jaburetox displays a membrane-disruptive ability on unilamellar lipid vesicles (Barros et al. 2009) and that both JBURE-I and Jaburetox are able to insert themselves into artificial lipid planar bilayers to form cation-selective ion channels (Piovesan et al. 2014). Taken together, these data suggest that at least part of the mechanism of action of both JBURE-I and Jaburetox involves an interaction with membrane lipids, promoting cellular permeabilization in the target insects.

Considering its entomotoxic activity, JBURE-I displayed toxicity towards *Dysdercus peruvianus* (cotton stainer bug), *Oncopeltus fasciatus* (large milkweed bug), and *R. prolixus* (Follmer et al. 2004; Stanisçuaski et al. 2010; Defferrari et al. 2011), and the JBURE-II isoform was also active against *R. prolixus* (Mulinari et al. 2011). Jaburetox was toxic against *D. peruvianus* and *R. prolixus*, as well as *S. frugiperda, Blatella germanica* (German cockroach), and *Triatoma infestans* (kissing bug; vector of Chagas disease in humans) (Mulinari et al. 2007; Tomazetto et al. 2007; Stanisçuaski et al. 2010; Stanisçuaski and Carlini 2012).

Unlike the hexameric JBURE-I, the canatoxin jack bean urease isoform is a homodimer of 95 kDa subunits (Carlini and Guimarães 1981) that displays

insecticidal activity against Coleoptera and Hemiptera (Carlini and Grossi-de-Sá 2002). Canatoxin is at least as toxic to insects as α-amylase inhibitors, proteinase inhibitors, and some lectins, in addition to being 40-fold more potent than the lectin arcelin to the coleopteran *Z. subfasciatus* (Carlini and Grossi-de-Sá 2002). Additionally, canatoxin is highly potent against two economically important hemipteran pests, the cosmopolitan pest *Nezara viridula* (Southern green soybean stinkbug) and *D. peruvianus*, which are not susceptible to the insecticidal activity of the tested Cry toxins and have developed resistance to certain chemical pesticides (Carlini et al. 1997; Ferreira-da-Silva et al. 2000; Carlini and Grossi-de-Sá 2002; Stanisçuaski and Carlini 2012).

The soybean embryo-specific urease (SBU) was also active against *D. peruvianus*. Although JBURE-I was slightly less toxic to this insect than canatoxin, JBURE-I was still threefold more potent than SBU (Follmer et al. 2004).

The urease JBURE-I and its encrypted peptide Pepcanatox (and the corresponding recombinant peptide Jaburetox) detrimentally affect insect cells. Upon ingestion by the insect, JBURE-I reaches the posterior midgut, where it is processed by the insect's digestive enzymes, releasing Pepcanatox among other peptides. Pepcanatox is transported to the hemolymph, where it disrupts the transepithelial potential of the insect Malpighian tubules, thus interfering with diuresis by blocking secretion (Stanisçuaski and Carlini 2012). However, the proteolytic release of Pepcanatox is only part of the entomotoxic property of ureases. In addition to inhibiting the diuresis of insect Malpighian tubules, JBURE-I (but neither Pepcanatox nor Jaburetox) increases the frequency and amplitude of the serotonin-induced contractions of the anterior midgut and hindgut, detrimentally altering the insect's physiology (Stanisçuaski et al. 2010; Stanisçuaski and Carlini 2012). Furthermore, several other insect tissues, such as the salivary glands, heart, and dorsal vessel, whose functions are also coordinated by serotonin, may be equally negatively affected by JBURE-I. The ion channel activity of the urease JBURE-I, the recombinant Jaburetox, and three Jaburetox deletion mutants (either lacking the N-terminal region, C-terminal region, or central β-hairpin) were tested on planar lipid bilayers (Piovesan et al. 2014). All proteins formed well-resolved, highly cation-selective channels, demonstrating the capacity of JBURE-I and Jaburetox to permeabilize membranes through an ion channel-based mechanism (Piovesan et al. 2014).

The Jaburetox mutant lacking the central β-hairpin region was still able to disrupt liposomes and displayed an entomotoxic activity similar to that of wild-type Jaburetox (Martinelli et al. 2014). Jaburetox mutants lacking either the N- or C-terminus also disrupted liposomes. Nevertheless, while the wild-type Jaburetox was highly insecticidal, the mutant consisting of the N-terminal half-peptide preserved most of the wild-type entomotoxicity, whereas the mutant corresponding to the C-terminal half-peptide was not lethal (Martinelli et al. 2014). In conclusion, the N-terminal portion of Jaburetox apparently carries the most important entomotoxic domain. Despite the fact that the β-hairpin region likely interacts with insect membranes, it is not essential for the entomotoxicity of Jaburetox (Martinelli et al. 2014).

Recently, it was demonstrated that upon Jaburetox injection, *T. infestans* displayed uncoordinated movements of the antennae and legs, and the administration of Jaburetox to adult insects led to 100% mortality in less than 24 h (Galvani et al. 2015). It was found that Jaburetox immunolocalized in the insect's central nervous system and interacted with an UDP-*N*-acetylglucosamine-phosphorylase (UDP-GlcNAc-phosphorylase) in the brain of the kissing bug. Moreover, Jaburetox treatment impaired the insect's central nervous system through inhibitory effects on nitric oxide synthase (NOS) activity, resulting in a drastic decrease in the nitric oxide (NO) levels. Interestingly, glycosyl-inositol-phospholipids, which indirectly derive from the activity of UDP-GlcNAc-phosphorylase, are known to downregulate NO synthesis. Therefore, it is speculated that the binding of Jaburetox to the kissing bug UDP-GlcNAc-phosphorylase leads to an increase in production of glycosyl-inositol-phospholipids and the subsequent NOS inhibition in the central nervous system of the Jaburetox-treated bugs (Galvani et al. 2015). Together, the data indicated that the normal activity of the central nervous system of *T. infestans* is impaired by the entomotoxic urease-derived peptide Jaburetox.

It is crucial to understand the effect of ureases and their encrypted entomotoxic peptides upon target insects to further elucidate the mechanism of action of these entomotoxins and, ultimately, to allow the resulting knowledge to be applied to plant protection strategies against insect-pests. Because neither JBURE-I nor SBU were lethal to mice or rats upon high-dose intraperitoneal administrations (Follmer et al. 2004), and many edible plants (particularly legumes and Cucurbitaceae) are rich sources of ureases; this class of proteins may confer a food biosafety advantage to GM plants. Although there is no record of GM plants expressing plant ureases or their encrypted-derived peptides, these entomotoxins represent a promising biotechnological strategy for the development of GM crops with durable resistance to insect-pests.

Chitinases

The chitin present in the extracellular layer of insect exoskeletons and peritrophic membranes is an interesting target for pesticide action (Cohen 1993). In addition to lectins, which can interact with chitin monomers and interfere with insect chitin synthases, plants also produce chitin hydrolytic enzymes, the chitinases (Cohen 1993).

Chitinases catalyze the hydrolysis of chitin, which is composed of β-1,4-linked *N*-acetylglucosamine residues (Collinge et al. 1993; Nagpure et al. 2014). Plant chitinases are either endochitinases or exochitinases, depending on the specific cleavage site in the chitin target molecules. Chitinases are usually monomeric proteins with a molecular mass ranging from 25 to 35 kDa (Collinge et al. 1993).

Plant chitinases can be classified into four different groups according to their primary structure. (i) The class I chitinases consist of enzymes with an N-terminal cysteine-rich domain of approximately 40 amino acid residues and a highly conserved main structure. (ii) The class II chitinase group is composed of enzymes that

Table 3 Entomotoxic plant chitinases and defensins expressed in GM plants

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[a]
Brassica rapa	Defensin: BrD1	*Nilaparvata lugens*	*Oryza sativa*	Choi et al. 2009
Populus tremuloides	Chitinase: WIN6	*Leptinotarsa decemlineata*	*Solanum lycopersicum*	Lawrence and Novak 2006
Tephrosia villosa	Defensin: TvD1	*Spodoptera litura*	*Nicotiana tabacum*	Vijayan et al. 2013

[a]**Choi et al**. 2009, Mol Cells 28(2):131–137; **Lawrence et al**. 2006, Biotechnol Lett 28:593–599; **Vijayan et al**. 2013, J Pest Sci 86:337–344

do not have the cysteine-rich domain at the N terminus of the molecule, despite their high amino acid sequence identity with class I chitinases. (iii) The class III chitinases include the enzymes with no sequence similarities to the proteins from class I or class II, although they share the same biochemical properties. (iv) The class IV chitinase group is composed of enzymes that are very similar to the class I chitinases and contain the cysteine-rich domain, although they possess four deletions and, consequently, have 45–60 fewer amino acid residues than other classes of chitinase enzymes (Collinge et al. 1993).

Although most plant chitinases exhibit activity against phytopathogenic bacteria and fungi (Cletus et al. 2013; Nagpure et al. 2014), few have demonstrated activity towards insect-pests. It has been described that plant chitinases affect the peritrophic membrane of larval midguts, which contain a matrix composed of chitin inserted in a protein-carbohydrate layer. A chitinase isolate from poplar plants (*Populus trichocarpa*), denoted as WIN6, exhibited activity against Colorado potato beetle larvae (*Leptinotarsa decemlineata*) when introduced into tomato plants (Table 3). Two chitinases, denoted LA-a and LA-b, identified in the latex of mulberry (*Morus* sp.) were active against *D. melanogaster* (Kitajima et al. 2010). When *D. melanogaster* larvae were fed with LA-a and LA-b in an artificial diet, 80 % and 40 %, respectively, of the insects were dead after 6 days (Kitajima et al. 2010). These observations point to the potential of the plant chitinases LA-a and LA-b against insects that are agricultural pests.

Proteases

Proteases, also referred to as peptidases or proteinases, are enzymes that are found in animals, plants, bacteria, archaea, and viruses, and hydrolyze the covalent bonds between the amino acids within a polypeptide chain. Some plant proteases have evolved as a form of protection against herbivorous insect-pests. Nevertheless, even proteases that have not evolved to act as entomotoxins can still have an insecticidal effect when they are ectopically administered within an insect-pest (Harrison and Bonning 2010). Some plant proteases deleteriously target the insect peritrophic matrix, which is composed of a net of chitin fibrils linked to glycoproteins and

proteoglycans and is located within the midgut of most insects. The disruption of this barrier increases the vulnerability of the insect's midgut to the entomotoxic molecules (Harrison and Bonning 2010).

There are few reported proteases with activity against insect-pests. Among them, a papain-like cysteine protease called Mir1-CP was identified in maize lines resistant to *S. frugiperda* (Jiang et al. 1995; Pechan et al. 1999; Lopez et al. 2007). Insect larvae fed on GM plant calluses expressing Mir1-CP exhibited growth inhibition (Pechan et al. 2000) and microscopic cracks/perforations in their gut matrix (Pechan et al. 2002). Moreover, purified recombinant Mir1-CP could degrade the peritrophic matrix of *S. frugiperda* and other insect species (Mohan et al. 2006), kill lepidopteran larvae, and enhance the toxicity of Bt Cry toxins (Mohan et al. 2008).

A protease-denoted papain, which is present in the latex of papaya (*Carica papaya*), and another cysteine protease called ficin, which is present in wild fig (*Ficus virgata*), retarded the growth of larvae of three different lepidopteran species, namely, *Mamestra brassicae* (cabbage moth), *Samia ricini* (Indian eri silkmoth), and *Spodoptera litura* (tobacco cutworm) (Konno et al. 2004).

Therefore, plant proteases represent a group of unexplored but promising agents for the development of insect-resistant GM plants (Harrison and Bonning 2010).

Inhibitors of Insect Digestive Enzymes: Plant Strategies to Block Pests' Metabolic Pathways

The insect digestive tract can be divided into the foregut, midgut, and hindgut. Most digestion occurs in the midgut, where a wide variety of enzymes have been identified, including abundant proteases and amylases. Plants have evolved mechanisms to block the insect's digestive enzymes through the production of proteinaceous protease and α-amylase inhibitors, which are discussed below.

Protease Inhibitors

Plant protease inhibitors (PIs) are part of the plants' innate defense system, as they inactivate the digestive proteases from herbivore insects. Due to the inhibition exerted upon the insect's digestive enzymes, plant PIs are deleterious to several insect-pests. Plant PIs compete with the substrate for the active site of the enzymes and interact with the proteases with a very low dissociation constant. Numerous plant PIs have been reported and the information is compiled in the Plant PIs database (http://plantpis.ba.itb.cnr.it/) (Consiglio et al. 2011). Plant PIs have been identified for all four classes of proteases, including serine, cysteine, aspartyl, and metalloproteinases, with the majority of PIs belonging to the serine PIs (Dang and Van Damme 2015). Two of the best-studied plant serine PIs are the Kunitz-type and the Bowman-Birk inhibitors. Kunitz-PIs are approximately 20 kDa and generally have low cysteine content and one active site, while Bowman-Birk-PIs are

approximately 9 kDa and usually have high cysteine content and two active sites (Dang and Van Damme 2015).

Numerous GM plants overexpressing plant PIs have been developed to increase plant resistance to insect-pests (Table 4). Nevertheless, the success of GM plants expressing PIs for insect control is hindered by the rapid adaptation of insect-pests to the plant PIs (Jongsma and Beekwilder 2011; Macedo et al. 2015b; Zhu-Salzman and Zeng 2015). The coevolution of phytophagous insects and their host plants has led to sophisticated physiological responses of insects to dietary PIs. The mechanisms underlying the flexibility of insect digestion to plant PIs are poorly understood. It has been suggested that the N- and C-termini of plant PIs bind to insect cell receptors to antagonize peptide hormone-regulated protease production (Jongsma and Beekwilder 2011).

Transgene stacking/pyramiding may be applied to enhance the efficacy of PIs in the GM plant context. For instance, the combined use of the potato PI StPin1A and the tobacco PI NaPI in GM cotton increased the resistance to the bollworm *Helicoverpa armigera* in both laboratory and field conditions (Table 4).

α-Amylase Inhibitors

α-Amylases (α-1,4-glucan-4-glucanohydrolases) belong to a class of digestive enzymes that catalyze the hydrolysis of the α-D-(1,4)-glucan linkages of starch, glycogen, and various other related carbohydrates (Franco et al. 2002). Insect α-amylases convert starch into oligosaccharides, which are further hydrolyzed to glucose by α-glucosidase, resulting in the production of a rich source of energy (Kaur et al. 2014).

Proteinaceous α-amylase inhibitors (α-AIs) occur naturally in several edible plants and are particularly abundant in legumes and cereals (Franco et al. 2002). When insect α-amylases are inhibited by plant α-AIs, the pest's nutrition is impaired, its growth and development are retarded, and eventually death occurs due to starvation (Kaur et al. 2014). To be effective, a plant α-AI must (i) substantially inhibit the insect α-amylases at a low concentration and at the same pH of the insect gut and (ii) be resistant to insect gut proteases. Furthermore, for biotechnological applications of α-AIs against insect-pests, the plant α-AIs should (i) be specific to their target α-amylase, (ii) not interfere with the action of the endogenous α-amylases involved in germination, and (iii) lack activity against mammalian α-amylases. These considerations should be taken into account when designing α-AI-based GM plant strategies against insect-pests (Kaur et al. 2014).

α-AIs have been characterized from different accessions of the common bean (*Phaseolus vulgaris*), including the white, red, and black kidney beans. The best-characterized isoform, known as α-AI-1, was cloned and identified as an α-AI homologous to plant lectins (Franco et al. 2002). A second variant of α-AI, called α-AI-2, is found in wild accessions of common bean. These two allelic α-AIs have diverse inhibition specificities, as α-AI-1 inhibits the α-amylases of the *C. maculatus* and Adzuki bean weevil *Callosobruchus chinensis*, but it does not

Table 4 Entomotoxic plant protease inhibitors (PIs) expressed in GM plants

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[c]
Glycine max	(NN)[a]	*Clostera anastomosis; Lymantria dispar*	*Populus* sp	Confalonieri et al. 1998
	Kunitz trypsin inhibitor	*Nilaparvata lugens*	*Oryza sativa*	Lee et al. 1999
	(NN)	*Spodoptera litura*	*Nicotiana tabacum*	McManus et al. 1999
	Kunitz inhibitor	*Spodoptera littoralis*	*Nicotiana tabacum*	Marchetti et al. 2000
			Solanum tuberosum	
Hordeum vulgare	CMe	*Sitotroga cerealella*	*Triticum aestivum*	Altpeter et al. 1999
	(NN)	*Sitophilus oryzae*	*Oryza sativa*	Alfonso-Rubi et al. 2003
Ipomoea batatas	(NN)	*Spodoptera litura*	*Nicotiana tabacum*	Yeh et al. 1997
	(NN)	*Pieris conidia; Plutella xylostella*	*Brassica oleracea*	Ding et al. 1998
Nicotiana attenuata	Threonine deaminase	*Manduca sexta*	*Nicotiana attenuata*	Kang et al. 2006
Nicotiana alata	NaPI	*Helicoverpa armigera*	*Nicotiana tabacum*	Charity et al. 1999
	(NN)	*Epiphyas postvittana*	*Malus domestica*	Maheswaran et al. 2007
Nicotiana alata and *Solanum tuberosum*	NaPI + StPin1A[b]	*Helicoverpa armigera*	*Gossypium hirsutum*	Dunse et al. 2010
Nicotiana attenuata	PI-II	*Manduca sexta*	*Nicotiana attenuata*	Zavala et al. 2004
Oryza sativa	(NN)	*Chrysomela tremulae*	*Populus* sp	Leplé et al. 1995
	OCII	*Leptinotarsa decemlineata*	*Solanum tuberosum*	Cingel et al. 2015
Psophocarpus tetragonolobus	(NN)	*Chilo suppressalis*	*Oryza sativa*	Mochizuki et al. 1999
Solanum lycopersicum	Arginase	*Manduca sexta*	*Solanum lycopersicum*	Chen et al. 2005
Solanum tuberosum	PI-I	*Manduca sexta*	*Nicotiana tabacum*	Johnson et al. 1989
	(NN)	*Chrysodeixis erisioma*		McManus et al. 1994
	PI-II	*Sesamia inferens*	*Oryza sativa*	Duan et al. 1996
	PI-II CPI	*Heliothis obsoleta; Liriomyza trifolii*	*Solanum lycopersicum*	Abdeen et al. 2005

(*continued*)

Table 4 (continued)

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[c]
	(NN)	*Chilo suppressalis*	*Oryza sativa*	Bu et al. 2006
	PINII	*Pieris rapae; Plutella xylostella*	*Brassica campestris*	Zhang et al. 2012
Vigna unguiculata	CpTI	*Manduca sexta*	*Solanum lycopersicum*	Hilder et al. 1987
		Multiple species *Otiorhynchus sulcatus*	*Malus domestica Fragaria sp.*	James et al. 1992; Graham et al. 1997
		Chilo suppressalis; Sesamia inferens	*Oryza sativa*	Xu et al. 1996
		Lacanobia oleracea	*Solanum tuberosum*	Gatehouse et al. 1997
		Spodoptera litura	*Nicotiana tabacum*	Sane et al. 1997
		Helicoverpa armigera	*Gossypium hirsutum*	Li et at. 1998
		Pieris rapae	*Brassica oleracea*	Lu et al. 2005
		Sitotroga cerealella	*Triticum aestivum*	Bi et al. 2006
Zea mays and *Solanum tuberosum*	MPI + PCI[b]	*Chilo suppressalis*	*Oryza sativa*	Quilis et al. 2014

[a]**(NN)** = No name was given to the insecticidal protein

[b]Pyramided and fused genes expressed within the same GM plant line

[c]**Abdeen et al**. 2005, Plant Mol Bio 57:189–202; **Alfonso-Rub et al**. 2003, Transgenic Res 12:23–31; **Altpeter et al**. 1999, Mol Breed 5:53–63; Bi et al. 2006, Euphytica 151:351–360; **Bu et al**. 2006, J Integr Plant Biol 48:732–739; **Charity et al**. 1999, Mol Breed 5:357–365; Chen et al. 2005, Proc Natl Acad Sci U S A 102:19237–19242; **Cingel et al**. 2015, Transgenic Res 24 (4):729–740; **Confalonieri et al**. 1998, Mol Breed 4:137–145; **Ding et al**. 1998, Plant Cell Rep 17:854–860; **Duan et al**. 1996, Nat Biotechnol 14:494–498; **Dunse et al**. 2010, Proc Natl Acad Sci U S A 107:15011–15015; **Gatehouse et al**. 1997, Mol Breed 3(1):49–63; **Graham et al**. 1997, Ann Appl Biol 131(1):133–139; **Hilder et al**. 1987, Nat 330:160–163; **James et al**. 1992, Phytoparasitica 20(1):S83-S87; **Johnson et al**. 1989, Proc Natl Acad Sci U S A 86:9871–9875; **Kang et al**. 2006, Plant Cell 18:3303–3320; **Lee et al**. 1999, Mol Breed 5:1–9; **Leplé et al**. 1995, Mol Breed 1:319–328; **Li et al**. 1998, Acta Gossypii Sinica 10:237–243; **Lu et al**. 2005, Afr J Biotechnol 4:45–49; **Maheswaran et al**. 2007, Plant Cell Rep 26:773–782; **Marchetti et al**. 2000, Theor Appl Genet 101:519–526; **McManus et al**. 1994, Transgenic Res 3:50–58; **Mochizuki et al**. 1999, Entomol Exp Appl 93:173–178; **Quilis et al**. 2014, Plant Biotechnol J 12(3):367–377; **Sane et al**. 1997, Curr Sci 72:741–747; **Xu et al**. 1996, Mol Breed 2:167–173; **Yeh et al**. 1997, Plant Cell Rep 16:696–699; **Zavala et al**. 2004, Plant Physiol 134:1181–1190; **Zhang et al**. 2012, Breed Sci 62(2):105–112

inhibit the *Zabrotes subfasciatus* bruchid α-amylases (Ishimoto and Kitamura 1989; Feng et al. 1996). In contrast, α-AI-2 does not inhibit the α-amylases from *Callosobruchus* spp, but it does inhibit the *Z. subfasciatus* α-amylases (Grossi-de-Sá et al. 1997; Silva et al. 2001). Later, it was described that α-AI-1 could also inhibit the enzymes from the pea weevil (*Bruchus pisorum*), the Western corn rootworm (*Diabrotica virgifera*), the coffee berry borer (*Hypothenemus hampei*), and the mealworm beetle larvae (*Tenebrio molitor*) (Table 5; Nahoum et al. 2000; Valencia et al. 2000; Titarenko and Chrispeels 2000; Valencia-Jiménez et al. 2008).

Two other bean α-AIs were also studied. The *P. vulgaris* chitinolytic α-amylase inhibitor (PvCAI) exhibited inhibitory activity against the larval *Z. subfasciatus* α-amylases and no activity against mammalian α-amylases (Dayler et al. 2015); the α-AIs present in scarlet runner bean (*Phaseolus coccineus*) were active against *H. hampei* α-amylase (Valencia et al. 2000; Valencia-Jiménez et al. 2008).

The α-AI BIII from rye (*Secale cereale*) was active against the cotton boll weevil (*Anthonomus grandis*) (Oliveira-Neto et al. 2003). The larvae of the coleopteran pests *Acanthoscelides obtectus* and *Z. subfasciatus* were equally susceptible to BIII (Dias et al. 2005). Nevertheless, BIII did not inhibit the activity of porcine pancreatic α-amylase (Dias et al. 2005).

To reach the active mature form composed of two noncovalently bound glycosylated α- and β-subunits, common bean α-AIs must undergo different post-translational modifications, such as proteolysis and the clipping of the residues at the C-terminus of the α-AI-1 β-subunits. α-AI-2 displays similar posttranslational modifications as α-AI-1, although they have different glycosylation patterns. Hence, both mature α-AI-1 and α-AI-2 have a heterotetrameric structure of two α-subunits and two β-subunits and are highly glycosylated.

The α-AI from amaranth (*Amaranthus hypochondriacus*) seeds (Chagolla-Lopez et al. 1994; Franco et al. 2002) is currently the smallest reported proteinaceous α-AI, with just 32 residues and three disulfide bonds. The amaranth α-AI (AAI) possesses a knottin fold, three antiparallel β-strands, and disulfide topology. AAI specifically inhibits the α-amylases from *Prostephanus truncatus* and *Tribolium castaneum* but is inactive against mammalian α-amylases (Chagolla-Lopez et al. 1994).

α-AIs from plants of the cereal family (Franco et al. 2002) are composed of approximately 140 amino acids linked by five disulfide bonds. The wheat α-AI, denoted as 0.19, is the most studied α-AI from the cereal family. Earlier studies showed that 0.19 is able to inhibit the enzymes of several insect-pests, including *A. obtectus, C. maculatus, D. virgifera, Lygus lineoralis, Sitophilus oryzae, T. molitor, T. castaneum*, and *Z. subfasciatus* (Sanchez-Monge et al. 1989; Feng et al. 1996; Franco et al. 2000; Titarenko and Chrispeels 2000).

Some cereal α-AIs are monomeric, such as the wheat α-AIs 0.28, WRP25, WRP26, and WRP27. In an in vitro assay, 0.28 has demonstrated activity against the *T. molitor* α-amylase (Sanchez-Monge et al. 1989). Moreover, the wheat peptides WRP25, WRP26, and WRP27 were able to inhibit the α-amylases from *C. maculatus, S. oryzae, T. molitor, T. castaneum*, and *Z. subfasciatus* (Feng et al. 1996; Franco et al. 2000).

Table 5 Entomotoxic plant α-amylase inhibitors (α-AI) in GM plants

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[b]
Phaseolus vulgaris	α − AI1	*Tenebrio molitor*	*Nicotiana tabacum*	Altabella and Chrispeels 1990
		Callosobruchus chinensis; Callosobruchus maculatus; Bruchus pisorum	*Pisum sativum*	Shade et al. 1994; Schroeder et al. 1995; Morton et al. 2000
		Hypotheneumus hampei	*Coffea arabica*	Barbosa et al. 2010
		Callosobruchus chinensis; Callosobruchus maculatus	*Cicer arietinum*	Sarmah et al. 2004; Ignacimuthu and Prakash 2006; Lüthi et al. 2013
		Callosobruchus chinensis; Callosobruchus maculatus	*Vigna unguiculata*	Solleti et al. 2008; Lüthi et al. 2013
		Callosobruchus analis; Callosobruchus chinensis	*Vigna angularis*	Ishimoto et al. 1996
	α − AI2	*Bruchus pisorum*	*Pisum sativum*	Morton et al. 2000
	α − AI1 + Cry1Ac/b[a]	*Helicoverpa armigera*	*Cicer arietinum*	Acharjee and Sarmah 2013
	α − AI1 + Cry2Aa[a]	*Callosobruchus chinensis; Callosobruchus maculatus*		
Phaseolus coccineus	α-AI-Pc1	*Hypotheneumus hampei*	*Nicotiana tabacum*	Pereira et al. 2006

[a]Pyramided genes within the same GM plant line

[b]**Acharjee and Sarmah** 2013, Plant Sci 207:108–116; **Altabella and Chrispeels** 1990, Plant Physiol 93(2):805–810; **Barbosa et al**. 2010, BMC Biotechnol 10:44–51; **Ignacimuthu and Prakash** 2006, J Biosci 31:339–345; **Ishimoto et al**. 1996, Entomol Exp Appl 79:309–315; **Lüthi et al**. 2013, Bull Entomol Res 103:373–381; **Morton et al**. 2000, Proc Natl Acad Sci U S A 97:3820–3825; **Pereira et al**. 2006, Phytochem 67:2009–2016; **Sarmah et al**. 2004, Mol Breed 14:73–82; **Schroeder et al**. 1995, Plant Physiol 107(4):1233–1239; **Shade et al**. 1994, Nat Biotechnol 12:793–796; **Solleti et al**. 2008, Plant Cell Rep 27:1841–1850

Thaumatin-like α-AIs (Franco et al. 2002) are proteins with molecular masses of approximately 20 kDa and have significant sequence homology to pathogenesis-related 5 (PR-5) proteins, also known as thaumatins. The best-characterized thaumatin-like α-AI is zeamatin, a bifunctional α-AI from maize. The structure of

zeamatin is stabilized by eight disulfide bonds. Zeamatin inhibits porcine pancreatic trypsin and the digestive α-amylases of the insects *T. castaneum*, *Sitophilus zeamais*, and *Rizopherta dominica* (Schimoler-O'Rourke et al. 2001).

The production of GM plants expressing α-AIs is an attractive and alternative approach to the use of chemical pesticides. There are various reports of GM plants expressing the common bean α-AI-1 that are effective against the target insect-pests (Kaur et al. 2014). When introduced into *N. tabacum*, the bean α-AI-1 was active against *T. molitor* (Table 5). Furthermore, a GM pea (*Pisum sativum*) expressing α-AI-1 under a strong seed promoter was effective against *B. pisorum* (Table 5), *C. chinensis*, and *C. maculatus* (Table 5). Additionally, a GM pea expressing the common bean α-AI-2 was toxic to *B. pisorum* (Table 5). When introduced into chickpea *C. arietinum*, the common bean α-AI-1 showed high insecticidal effects against the larvae of the two species of bean beetles (*C. maculatus* and *C. chinensis*) (Table 5). A GM cowpea (*Vigna unguiculata*) expressing the same α-AI exhibited similar effects (Table 5). Moreover, GM chickpeas expressing either the Cry1Ac/b or the Cry2Aa and the common bean α-AI-1 were resistant to *H. armigera* and bruchids (*C. chinensis* and *C. maculatus*), respectively (Table 5). A GM Adzuki bean (*Vigna angularis*) expressing the common bean α-AI-1 displayed resistance to the bruchids *Callosobruchus analis* and *C. chinensis* (Table 5). Coffee plants (*Coffea arabica*) were also transformed with common bean α-AI-1 and demonstrated significant inhibitory activity towards *H. hampei* (Table 5). Additionally, when the α-AI-Pc1 from *P. coccineus* was introduced into tobacco plants, it inhibited 65 % of the digestive *H. hampei* α-amylases (Table 5).

A main challenge for the use of α-AIs in GM plants for protection against insects is the fact that the targeted insect-pests may develop resistance to the inhibitor. Therefore, efforts must be concentrated on the identification of plant α-AI genes that are resistant to the proteases of different target insects (Kaur et al. 2014).

Plant Peptides: Small Molecules for the Control of Insect-Pests

Defensins

Plant defensins are small cationic peptides, ranging from 45 to 54 amino acid residues, stabilized by 3–4 disulfide bridges and a molecular mass of approximately 5 kDa (Lacerda et al. 2014). In general, the three-dimensional structure of defensins is characterized by an α-helix followed by three antiparallel β-sheets (Lacerda et al. 2014). To date, several defensins have been isolated from plant leaf, stem, root, and endosperm tissues and exhibit a wide range of activities, such as antibacterial, antifungal, and insecticidal effects (Lacerda et al. 2014).

Plant defensins that exhibit pesticide activity are a relatively recent field of scientific investigation compared to other types of plant insecticidal proteins. Plant defensins primarily inhibit insect enzymes, particularly α-amylases and proteases, making them part of the previously discussed class of plant insecticidal proteins, i.e., inhibitors of the insect digestive enzymes.

The first plant defensin with insecticidal activity was isolated from sorghum (*Sorghum bicolor*) (Bloch and Richardson 1991). It exhibited inhibitory activity against the α-amylases of the insects *Periplaneta americana* and *S. americana*, while it had no effect upon the mammalian enzymes (Bloch and Richardson 1991). The defensin NaD1 isolated from *Nicotiana alata* exhibited insecticidal activity towards the lepidopterans *H. armigera* and *Helicoverpa punctigera* (Lay et al. 2003). Further analyses on the expression of NaD1 in GM tobacco showed an enhanced mortality rate and detrimental effects on development of the same insect species (Lay et al. 2003). A defensin from papaya (*C. papaya*) exhibited activity against the α-amylases from the *C. maculatus* bruchid (Farias et al. 2007).

The defensin isolated from seeds of mung bean (*Vigna radiata*), denoted as VrD1, has been thoroughly studied in terms of its structure and function. The VrD1 cDNA was isolated from a bruchid-resistant mung bean, and the corresponding protein was expressed in a yeast system (Chen et al. 2004). The recombinant VrD1 expressed in yeast was active against *C. chinensis* in bioassays with artificial mung bean seeds (Chen et al. 2004).

The VuD1 defensin, which was isolated and cloned from cowpea, was shown to inhibit the α-amylases from the *A. obtectus* and *Z. subfasciatus* insects, although it had no activity against *C. maculatus* (Pelegrini et al. 2008). Moreover, VuD1 inhibited porcine pancreas amylases at low levels, while it had no effect upon the human salivary enzymes (Pelegrini et al. 2008). Further studies have shown that the recombinant VuD1 protein is able to inhibit the α-amylases of the weevil *C. maculatus* at micromolar concentrations, without affecting the mammalian enzymes (Santos et al. 2010). Molecular modeling analyses helped to elucidate the interaction between VuD1 and the α-amylase ZSA from *Z. subfasciatus*. The salt-bridge interaction between Lys_1 from VuD1 with Glu_{240} in the active site of ZSA seemed to be one of the first steps in enzyme inhibition. The positively charged amino acid residue Lys_1 from VuD1 could also form a hydrogen bond with Asp_{305} in the enzyme's catalytic site (Pelegrini et al. 2008). Furthermore, the C-terminal amino acid residues from VuD1 interacted with the amino acids present in the active site of ZSA. In contrast to VrD1, the data demonstrated that the enzymatic inhibition by VuD1 occurs by ionic and hydrogen bond formation within the catalytic site of insect α-amylases, with the VuD1 defensin using the residues located at its N- and C-termini instead of loop 1 and loop 2 (Pelegrini et al. 2008).

The defensin TvD1 from the weedy legume *Tephrosia villosa* was mutated in and around the β2–β3 loop region through in vitro mutagenesis, generating the variant alpha-TvD1 (Vijayan et al. 2012). Both wild-type TvD1 and alpha-TvD1 exhibited inhibitory activity against the α-amylase of *T. molitor*, with the latter showing enhanced activity (Vijayan et al. 2012). Furthermore, TvD1 was overexpressed in tobacco, and a high expression plant line exhibited strong in vivo antifeedant activity against the larvae of *S. litura* (Table 3).

Although there are several reports on insecticidal plant defensins, few studies have investigated the use of these peptides for developing GM plants that are resistant to insect-pests. The defensin BrD1 isolated from turnip (*Brassica rapa*) was evaluated in GM rice cultivars (Table 3). GM rice lines expressing BrD1

exhibited increased resistance towards the attack of *N. lugens* compared to the nontransformed plants (Table 3).

Cyclotides

Plant cyclotides belong to a peptide group that is highly similar to defensins. They are cationic peptides with a low molecular mass and are approximately 30 amino acid residues in length; however, unlike defensins, they lack N- and C-termini (Pelegrini et al. 2007; the reader is also referred to ▶ Chap. 9, "Moonlighting Toxins: Ureases and Beyond." The three-dimensional structure of plant cyclotides is composed of a head-to-tail backbone formed by six conserved cysteine residues that characterize a knot motif (Pelegrini et al. 2007).

Currently, many cyclotides from plant sources have been isolated and characterized. Their described functions include antibacterial, antiviral, antitumoral, insecticidal, and hemolytic activities (Pelegrini et al. 2007; the reader is also referred to ▶ Chap. 18, "Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities.")

The first insecticidal plant cyclotide was described in 2001 in experiments using the cyclotide kalata B1, which was isolated from the African plant *Oldenlandia affinis*. Kalata B1 was active against the lepidopteran *H. punctigera* (Jennings et al. 2001). When added to an artificial diet, kalata B1 was able to decrease the growth and development of *H. punctigera* larvae, although the cyclotide did not affect the activity of any of the insect-pest's digestive enzymes. Therefore, it was suggested that the mechanism of action of kalata B1 was physical damage to membranes of the insect's midgut (Jennings et al. 2001). Recent studies focused on the expression of kalata B1 in GM *Nicotiana benthamiana* and on understanding how this peptide cyclized. Three highly conserved regions, which are essential for the proper posttranslational modifications of cyclotides, were identified at the C-terminus of kalata B1 (Conlan et al. 2012).

In addition to kalata B1, the insecticidal activity of kalata B2 was also evaluated, indicating that both *O. affinis* cyclotides were able to inhibit the growth and development of *H. armigera* larvae (Jennings et al. 2005). An artificial diet containing either kalata B1 or kalata B2 could inhibit *H. armigera* growth. Although there are slight differences between the structures and characteristics of both peptides, their activities against insect-pests were very similar. Nevertheless, the mechanisms of action of kalata B1 and kalata B2 against insect-pests are yet to be determined. The membrane disruption caused by these circular peptides may occur either by pore formation or simply by a generalized disturbance of the membrane structure. Although it is known that the cyclotides kalata B1 and B2 can form tetramers and octamers, it cannot yet be assumed that a multimer of these cyclotides is mandatory to disturb the insects' membranes (Jennings et al. 2005). An NMR spectroscopy analysis of kalata B2 demonstrated that its oligomer form is

not related to the insertion of this peptide into the membrane, probably representing a way of preventing self-toxicity in the plant (Rosengren et al. 2013).

Further investigations demonstrated that kalata B1 forms pores with channel-like activities in the membrane of insect midguts. Assays revealed that the kalata B1 inserts into the lipid bilayers of the cell membrane through hydrophobic interactions between its nonpolar amino acids and the hydrophobic core of the membrane to form oligomers, either tetramers or octamers. This contact increases membrane permeability, leading to pore formation and facilitating the leakage of the vesicular contents (Huang et al. 2009). Moreover, the size of the pores formed ranged from 41 to 47 Å in diameter, confirming that they correspond to typical ion channels.

When *H. armigera* larvae fed on an artificial diet with high concentrations of kalata B1, their food consumption was very low, indicating that the inhibition of larvae development was due to the lack of nutrient intake rather than a toxic effect of the cyclotide (Barbeta et al. 2008). Nevertheless, when a low concentration of kalata B1 was added to the diet, the larvae consumed more food, but their development was still repressed. This result suggested that while nutrient intake was reduced, it was not the only cause for growth inhibition (Barbeta et al. 2008). Therefore, light and electron microscopy analyses were performed to investigate whether the membrane in the insect midgut was disrupted and the mechanism by which kalata B1 damaged the cell membranes. The microscopic images demonstrated that the cells' microvilli were disrupted and the epithelial layer was obstructed by the granular components released from the lysed cells (Barbeta et al. 2008). The cells ruptured due to pore formation, leading to swelling and subsequent lysis. As this mechanism of action is very similar to that of the Cry toxins and Vip3A from *B. thuringiensis*, it was suggested that tetrameric or octameric cyclotides may cause pore formation (Barbeta et al. 2008). However, this hypothesis was disproven when further studies revealed that the formation of tetrameric cyclotides is dependent on the concentration and occurs as a self-defense mechanism against the toxic cyclotides that plants produced endogenously (Rosengren et al. 2013). Furthermore, it was proposed that plant cyclotides bind to phosphatidylethanolamine-containing lipids, which indicates that these peptides participate in specific interactions with the cell membrane (Kamimori et al. 2005).

Another cyclotide isolated from blue pea (*Clitoria ternatea*), denoted as finotin, caused 100 % mortality of the *Z. subfasciatus* and *A. obtectus* insect-pests when added to an artificial diet (Kelemu et al. 2004). Recently, a cyclotide from the Brazilian Savannah Rubiaceae flower plant *Palicourea rigida*, called paragidin-BR1, was isolated and resulted in 60 % mortality of *Diatraea saccharalis* larvae after 15 days in an artificial diet assay. Moreover, in vitro assays demonstrated the efficacy of paragidin-BR1 against the SF-9 *S. frugiperda* cell line at micromolar concentrations of the cyclotide (Pinto et al. 2012).

There is potential for the use of cyclotides in future applications of GM plants for protection against insects. However, to date, GM plants expressing cyclotide genes for resistance against insect-pests have not been reported.

Conclusions and Future Directions

Challenges and Alternatives to Develop Durable Plant Resistance to Insect Pests

Insect-pests coevolve with their host plants, and the complex mutual attack-defense strategies are dynamic; hence, insects continuously counteract the resistance of plants. For instance, the insect's digestive enzymes frequently adapt to plant toxins, such as PIs, as previously discussed in this chapter (Zhu-Salzman and Zeng 2015). These adaptive counteractive measures pose barriers to GM plant-based insect control approaches.

Therefore, multiple mechanisms of resistance in GM crops are increasingly desirable through the use of various strategies for plant protection against insect-pests. The use of proteins from various sources with different mechanisms of action can produce a synergistic effect against insect-pests and may be an alternative to Bt application (Chougule and Bonning 2012). In this context, the use of entomotoxic proteins from plant sources is highly encouraged.

Additionally, attention must be given to the gene promoter that drives entomotoxin expression in the GM plant. The use of tissue-specific gene promoters to direct the expression of the entomotoxin to the sites of attack by the insect-pest may be a determinant in developing a resistant GM plant. For instance, the expression of the ASAL garlic lectin driven by phloem-specific promoters in GM tobacco resulted in resistance to the phloem-feeding aphid *M. nicotianae* and resulted in the resistance of GM rice to the sap-sucking hopper *N. lugens* (Table 1). Furthermore, GM legume plants (pea, chickpea, cowpea, and Adzuki bean) that transgenically expressed α-AI-1 under a strong seed promoter were all effective against several seed-feeding beetles (Table 5).

The resistance of GM plants expressing Bt entomotoxins to insect-pests has been extensively studied and successfully applied in practice (Palma et al. 2014; James 2014). The concomitant use of Bt entomotoxins with entomotoxins from other nonbacterial sources, such as plant insecticidal proteins, may enhance the synergistic control of insects. However, in some cases, Bt entomotoxins exhibit low toxicity against sap-sucking insects (Chougule and Bonning 2012). This limitation may be due to the fact that the Bt toxins have not evolved to combat sap-sucking insects because these pests are not exposed to the toxins. First, Bt bacteria exist in the soil and on the surface of the foliage; hence, there was no selection for toxicity to insects that pierce into the leaves (Chougule and Bonning 2012). Second, the differences in the gut conditions that activate the Bt toxins (i.e., proteolytic enzymes and gut pH) between sap-sucking insects and chewing insects are aggravating issues for the low Bt toxicity against piercing pests (Chougule and Bonning 2012). This limitation of the use of Bt toxins to control phloem-feeding insects makes the choice of the gene promoter, driving the entomotoxin expression within the GM plant even more relevant.

Alternatively, GM or non-GM crops expressing resistance R genes to insects are used for protection against insect-pests. The R gene-mediated defense system

detects the presence of the insect avirulence Avr proteins and initiates the hypersensitive response (HR), which triggers cellular apoptosis within the attacked plant tissue. Nevertheless, the extremely high specificity of R-Avr interactions tremendously limits the range of action on different insect species and even on populations within the same species. In this case, the use of plant entomotoxins also increases the possibilities of developing durable, resistant GM plants.

Plants have evolved constitutive and induced secondary metabolites as a major barrier to herbivory. Examples of protective plant secondary metabolites include cyanogenic glycosides, glucosinolates, alkaloids, terpenoids, steroids, and phenolics (the reader is referred to ▶ Chaps. 11, "Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action," and ▶ 13, "Plant Cyanogenic Glycosides." Usually, these metabolites are small lipophilic molecules (SLMs) that may have similar activity to the currently used chemical insecticides (Birkett and Pickett 2014). The phenolic tannins are the most abundant secondary metabolites produced by plants and defend the leaves against insect herbivores by deterrence and/or toxicity (Barbehenn and Constabel 2011). Tannins have no effect on protein digestion in insect herbivores, but are rather prone to oxidation in their high pH guts, producing high levels of toxic reactive oxygen species (Barbehenn and Constabel 2011). Secondary plant metabolites are a valuable alternative for the development of plant resistance against insect-pests. Genetic engineering of secondary metabolite pathways has been performed to promote the production of entomotoxic SLMs and tannins by the GM plant (Barbehenn and Constabel 2011; Birkett and Pickett 2014). Nevertheless, engineering the secondary metabolic pathways is a strategy particularly complex and challenging.

The recently obtained genomic sequences of insect-pests provide the necessary target information for RNAi-based gene function analysis and for the potential applications of RNAi in pest control. Gene silencing through RNAi in GM plants combined with the use of entomotoxic proteins from plants and other sources enhance the potential for the development of durable GM plants that are resistant to insect-pests. MicroRNAs (miRNAs) have also been identified as important regulators of gene expression in animals and plants and can control diverse biological processes, including defense. Recently, the artificial miRNA (amiRs) technology has been explored to disrupt the specific pathways targeted by these miRNAs, and, when expressed in plants, amiRs could target and silence the invading insect's genes, consequently conferring insect resistance (Younis et al. 2014).

Additionally, innovative approaches for insect-pest control involve biotechniques such as protein engineering for the design of novel and more potent chimeric insecticidal proteins (through phage display, direct protein evolution, and in vitro mutagenesis) and gene pyramiding in a single GM crop (Table 1, 2, 4, and 5).

Future Directions for Durable Plant Resistance to Insect Pests

Apart from the GM plant approach, an alternative approach to the use of different plant entomotoxic proteins for plant protection against insect-pests may be through

nanotechnology, which has been intensively studied for the development of new biopesticide products. Using nanotechniques, plant entomotoxic proteins may be encapsulated in nanoparticles, thus providing biopesticides and even medicine, with controlled release at specific sites.

Hence, the use of entomotoxins from plant sources for the next generation of GM plants is a promising alternative for the future market. It is important to emphasize that the introduction of plant insecticidal genes in GM plants must be applied with other control methods/strategies, such as biological control, crop rotation, and the use of chemical pesticides in the context of integrated pest management (IPM).

In conclusion, the development of biosafe GM crops with durable resistance to insect-pests requires a continuous search for alternative target-specific molecules for gene stacking to prevent insect resistance under field conditions and deleterious effects on nontarget organisms, all in the context of the IPM scenario.

Cross-References

- ▶ Biotechnological Potential of Ribosome-Inactivating Proteins (RIPs)
- ▶ Cyclotides: Plant Defense Toxins
- ▶ General Mechanisms of Plant Defense and Plant Toxins
- ▶ Moonlighting Toxins: Ureases and Beyond
- ▶ Plant AB Toxins with Lectin Domains
- ▶ Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action
- ▶ Plant Cyanogenic Glycosides
- ▶ Plant Toxins as Sources of Drugs
- ▶ Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities
- ▶ Ribosome-Inactivating Proteins: An Overview
- ▶ Toxic but Exploitable Actions of Ribosome-Inactivating Proteins

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