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Plant Proteinaceous α -Amylase and Proteinase Inhibitors and Their Use in Insect Pest Control

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1. Introduction

During the evolution, plants have developed strategies to maintain favorable growth and also guarantee their survival. Enhancing the protective mechanisms, for example, is one of these strategies that allow them to successfully tolerate/resist insects, phytopathogenic microorganisms, and other unfavorable conditions (Jackson and Tailor, 1996; Malek and Dietrich, 1999; Stotz et al, 1999). Proteinaceous molecules such as α -amylase inhibitors (α -AI) and proteinase inhibitors (PIs), lectins, some hydrolyzing enzymes (e.g. b-1,3-glucanases and chitinases), and also antimicrobial peptides, are important part of protective mechanisms in plants (Fritig et al., 1998; Howe, 2008). Host plant resistance and natural plant products offer a potentially benign method for insect pest control. They are safe to the non-target beneficial organisms and human beings (Andow, 2008). This kind of plant resistance can be utilized as an economic means to reduce crop losses arising from insect pest. The wild accessions of wheat and barley do not have effective resistance and therefore plant traits that contribute to pest resistance need to be reinforced using new approaches. Thus, using the new control methods are needed such as α -amylase inhibitors, protease inhibitors, lectins and possibly δ -endotoxin (Sharma and Ortiz, 2000) to diminish reliance on insecticides.

In recent years, attentions have been focused on the idea of using digestive enzyme inhibitors that affect the growth and development of pest species (Mehrabadi et al., 2010, 2011, 2012). Inhibitors of insect α -amylase, proteinase and other plant proteins have already been demonstrated to be an important biological system in the control of insect pests (Chrispeels *et al.*, 1998; Gatehouse and Gatehouse, 1998; Morton et al., 2000; Valencia *et al.*, 2000; Carlini and Grossi-de-Sa', 2002; Svensson *et al.*, 2004; Barbosa et al., 2010). Different types of proteinaceous α -AIs are found in microorganisms, plants and animals. Cereals such as wheat, barley, rye, rice and sorghum contain small amylase inhibitors about 18 kDa in size (Abe *et al.*, 1993; Feng *et al.*, 1996; Yamagata *et al.*, 1998; Iulek *et al.*, 2000). There are different kinds of inhibitors that potentially are a good source of α -amylases inhibitors that could be used against insect pest (Franco *et al.* 2002; Svensson et al., 2004). They show

diverse structural differences thus causing different mode of actions and diverse specificity against target enzymes. Different α -amylase inhibitors have different modes of action against α -amylases for example inhibitors extracted from cereals and beans (*Phaseolus vulgaris*) have different molecular structures, leading to different modes of inhibition and different specificity against a diverse range of α -amylases. Specificity of inhibition is an important issue as the introduced inhibitor must not adversely affect the plant's own α -amylases or human amylases and must not change the nutritional value of the crop (Franco *et al.* 2002; Svensson *et al.*, 2004).

Proteinase inhibitors are capable of interfering with insect protein digestion by binding to digestive proteases of phytophagous insects, resulting in an amino acid deficiency thus affecting insect growth and development, fecundity, and survival (Lawrence and Koundal 2002; Oppert *et al.* 2003; Azzouz *et al.* 2005). They along with α -amylases inhibitors constitute major tools for improving the resistance of plants to insects. Transgenic plants expressing serine and cysteine proteinase inhibitors have shown resistance to some insect pest species including Lepidoptera and Coleoptera (De Leo *et al.* 2001; Falco and Silva-Filho 2003; Alfonso-Rubi *et al.* 2003). PIs are the products of single genes, therefore they have practical advantages over genes encoding for complex pathways and they are effective against a wide range of insect pests, i.e. transferring trypsin inhibitor gene from *Vigna unguiculata* to tobacco conferred resistance against lepidopteran insect species such as *Heliothis* and *Spodoptera*, and coleopteran species such as *Diabrotica* and *Anthonomus* (Hilder *et al.* 1987).

2. Insect α -amylases

α -Amylases (α -1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) are one of the most widely enzyme complexes encountered in animals, higher and lower plants, and microbes. Because of their importance in organism growth and development, these enzymes from different origins including bacteria, nematodes, mammals and insects have been purified and their physical and chemical properties characterized (Baker, 1991; Nagaraju and Abraham, 1995; Zoltowska, 2001; Rao *et al.*, 2002; Valencia *et al.*, 2000; Mendiola-Olaya *et al.*, 2000; Oliveira-Nato *et al.*, 2003 Mohammed, 2004; Bandani *et al.* 2009; Mehrabadi *et al.* 2009). Many phytophagous insects, like stored product insects, live on a polysaccharide-rich diet and are dependent on their α -amylases for survival (Mendola-Olaya *et al.* 2000; Boyd *et al.* 2002; Mehrabadi *et al.* 2011). They convert starch to maltose, which is then hydrolyzed to glucose by α -glucosidase. In insects, only α -amylases that hydrolyse α -1,4- glucan chains such as starch or glycogen have been found (Terra *et al.* 1999).

According to Terra and Ferreira (1994), insect α -amylases generally have molecular weights in the range 48–60 kDa, pI values of 3.5–4.0, and K_m values with soluble starch around 0.1%. pH optima generally correspond to the pH prevailing in midguts from which the amylases were isolated. Insect amylases are calcium-dependent enzymes, and are activated by chloride with displacement of the pH optimum. Activation also occurs with anions other than chloride, such as bromide and nitrate, and it seems to depend upon the ionic size (Terra and Ferreira, 1994).

Although the sequences of several insect α -amylases are known, the best characterized insect α -amylase whose 3D-structure has been resolved is *Tenebrio molitor* (TMA) α -

amylase. The three-dimensional model of TMA consists of a single polypeptide chain of 471 amino acid residues, one calcium ion, one chloride ion and 261 water molecules. The enzyme consists of three distinct domains, A (residues 1 to 97 and 160 to 379), B (residues 98 to 159) and C (residues 380 to 471) (Strobl et al, 1998). A representation of the overall polypeptide folds, as well as the location of the bound ions and the residues presumably involved in catalysis. Domain A is the central domain with $(\beta / \alpha)_8$ -barrels comprises the core of the molecule and also includes the catalytic residues (Asp 185, Glu 222 and Asp 287). Two other domains (Domains B and C) are opposite each other, on each side of domain A. The Ca^{2+} binding site in TMA is located at the interface of the domain A central β -barrel and domain B. This ion is important for the structural integrity of TMA. TMA has also chloride-binding site on the same side of the β -barrel as the catalytic and the calcium-binding site, in the vicinity of both. Insect α -amylases are closely related to mammalian α -amylases (Strobl et al., 1997). The most striking difference between mammalian and insect α -amylases is the presence of additional loops in the vicinity of the active site of the mammalian enzymes (Strobl et al., 1998a). α -Amylases are the most important digestive enzymes of many insects that feed exclusively on seed products during larval and/or adult life. When the action of the amylases is inhibited, nutrition of the organism is impaired causing shortness in energy.

3. Proteinaceous α -amylase inhibitors from plants

α -AIs are abundant in microorganisms, higher plants, and animals (Da Silva et al., 2000; Toledo et al., 2007). These organisms produce a large number of different protein inhibitors of α -amylases in order to regulate the activity of these enzymes. α -Amylase inhibitors can be extracted from several plant species including legumes (Marshall and Lauda, 1975; Ishimoto et al., 1996; Grossi-de-Sa et al., 1997) and cereals (Abe et al., 1993; Feng et al., 1996; Yamagata et al., 1998; Franco et al., 2000; Iulek et al., 2000). Diverse α -amylase inhibitors reveal different characteristics against various α -amylases (Franco et al., 2002). In Table 1 inhibitory activity of α AIs from different sources are reviewed. α -AIs are naturally used by plants as a defense mechanism against insect pests (Ishimoto et al., 1989; Kluh et al., 2005). Moreover, there is a great interest to use α -AIs for control of insect pest and also to use them for production of transgenic plants that are resistant against insect pest (Gatehouse et al., 1998; Chrispeels et al., 1998; Gatehouse and Gatehouse, 1998; Valencia et al., 2000; Yamada et al., 2001). Inhibitors to insect α -amylase have already been demonstrated to be an important biological system in the control of insect pests (Franco et al., 2002; Carlini and Grossi-de-Sa', 2002; Svensson et al., 2004). The expression of α -amylase inhibitors has been showed to be effective in transgenic plants. The expression of the cDNA encoding α AI-I into some plants such as pea (*Pisum sativum* L.) and azuki bean (*Vigna angularis* L.) against bruchid beetle pests (Coleoptera: Bruchidae) has been well documented for showing ability of these inhibitors to be used as plant resistance factors against some species of insect pests (Ishimoto et al., 1989; Yamada et al., 2001; Kluh et al., 2005). Pea and azuki transgenic plants expressing α -amylase inhibitors from common beans (α -AI) were completely resistant to the *Bruchus pisorum* and *Callosobruchus chinensis* weevils (Morton et al. 2000). Rye α -amylase inhibitor expressed in transgenic tobacco seeds (*Nicotiana tabacum*) caused 74% mortality in *Anthonomus grandis* first instar larvae when transgenic seed flour mixture used in artificial diet (Dias et al., 2010).

α-Amylase inhibitor	Plant origin	Target pest	Test condition	Reference
αBIII	<i>Secale cereale</i>	<i>Anthonomus grandis</i> <i>Acanthoscelides obtectus</i> , <i>Zabrotess subfasciatus</i> and	transgenic tobacco In vitro	(Dias et al., 2005, 2010)
Ric c 1 and Ric c 3	<i>Ricinus communis</i>	<i>Callosobruchus maculatus</i> , <i>Zabrotes subfasciatus</i> , <i>Tenebrio molitor</i>	Feeding assay	(Do Nascimento et al., 2011)
Baru seed extract	<i>Dipteryx alata</i>	<i>Callosobruchus maculatus</i>	In vivo	(Bonavides et al., 2007)
α-AI-1 and α-AI2	<i>Phaseolus coccineus</i>	<i>Hypothenemus hampei</i> <i>Tecia solanivora</i>	In vitro	(Valencia-Jiménez et al., 2008)
αAI-Pc1	<i>Phaseolus coccineus</i>	<i>Hypothenemus hampei</i>	Transgenic plant	(de Azevedo et al.,2006)
α-AI1, α-AI2	<i>Phaseolus vulgaris</i>	<i>coffee berry borer pest</i> <i>Callosobruchus maculatus</i> <i>Callosobruchus chinensis</i> <i>Zabrotes subfasciatus</i> <i>Sitophilus oryzae</i> <i>Acanthoscelides obtectus</i> <i>Cryptolestes ferrugineus</i> <i>Cryptolestes pusillus</i> <i>Oryzaephilus surinamensis</i> <i>Sitophilus granarius</i> <i>Tribolium castaneum</i> <i>T. castaneum</i> <i>Drosophila melanogaster</i> <i>Sarcophaga bullata</i> <i>Aedes aegypti</i> <i>Monomorium pharaonis</i> <i>Apis mellifica</i> <i>Venturia canescens</i> <i>Ephestia cautella</i> <i>E. elutella</i> <i>E. kuehniella</i> <i>Manduca sexta</i> <i>Ostrinia nubilalis</i> <i>Blattella germanica</i> <i>Liposcelis decolor</i> <i>Acheta domesticus</i> <i>Eurydema oleracea</i> <i>Graphosoma lineatum</i>	Transgenic plant In vivo	(Barbosa et al., 2010; Solleti et al., 2008; Nishizawa et al., 2007; Ignacimuthu, and Prakash, 2006; Kluh et al., 2005)
VuD1	<i>Vigna unguiculata</i>	<i>Acanthoscelides obtectus</i> and <i>Zabrotes subfasciatus</i>	In vitro	(Pelegrini et al., 2008)
VrD1	<i>Vigna radiata</i>	<i>Tenebrio molitor</i>	In silico	(Liu et al., 2006)
KPSI	<i>Vigna radiata</i>	<i>Callosobruchus Maculatus</i>	In vivo	(Wisessing, 2010)

α -Amylase inhibitor	Plant origin	Target pest	Test condition	Reference
DR1-DR4	<i>Delonix regia</i>	<i>Callosobruchus maculatus</i> <i>Anthonomus grandis</i>	In vivo	(Alves, 2009)
(AI)-1 and (AI)-1	<i>Pisum sativum</i>	<i>Bruchus pisorum</i>	Transgenic plant	(De Sousa-Majer, et al., 2007)
CpAI	<i>Carica papaya</i>	<i>Callosobruchus maculatus</i>	In vivo	(Farias, et al., 2007)
α -AIs from <i>Triticum aestivum</i>	<i>Triticum aestivum</i>	<i>Eurygaster integriceps</i> <i>Tenebrio molitor</i> <i>Rhyzopcrtha dominica</i> <i>Callosobruchus maculates</i>	In vitro In vivo	(Mehrabadi et al., 2010, 2012; Zoccatelli et al., 2007; Cinco-Moroyoqui et al., 2006; Amirhusin, etal., 2004)
0.19 AI 0.53 AI	<i>Triticum aestivum</i>	<i>Acanthoscelides obtectus</i>	In vivo	(Franco et al., 2005)
BIII	<i>Secale cereale</i>	<i>Acanthoscelides obtectus</i> , <i>Zabrotess subfasciatus</i> <i>Anthonomus grandis</i>	In vivo In vitro	(Dias, et al., 2005; Oliveira-Neto et al., 2003)
SPAI1-SPAI4	<i>Ipomoea batatas</i>	<i>Araecerus fasciculatus</i> <i>Sitophilus oryzae</i> <i>Cylas formicarius</i> <i>elegantulus</i> <i>Tribolium castaneum</i>	In vitro	(Rekha, et al., 2004)
TAI1,TAI2 C154, C178, C249, C439, C487	<i>Colocasia esculenta</i>	<i>Araecerus fasciculatus</i> <i>Sitophilus oryzae</i> <i>Cylas formicarius</i> <i>elegantulus</i> <i>Tribolium castaneum</i>	In vitro	(Rekha, et al., 2004)
α -PPAI and α -ZSAI	<i>Ficus</i> sp.	<i>Callosobruchus maculatus</i> <i>Zabrotes subfasciatus</i>	In vitro	(Bezerra et al., 2004)
PpAI	<i>Pterodon pubescens</i>	<i>Callosobruchus maculatus</i>	In vivo	(Silva et al., 2007)

Table 1. Plant α -amylase inhibitors and their activities against insect pests (literature review since 2002).

3.1 Plant α -amylase inhibitor classes

Based on structural similarity, there are six different proteinaceous α -amylase inhibitors with plant origin including lectin-like, knottin-like, CM-proteins, Kunitz-like, c-purothionin-like, and thaumatin-like (Richardson, 1990) (Table 2).

Inhibitor class	Plant origin	Target	Residues number (aa)	Names	References
Legum lectin-like	Common bean	Insect, Mammalian, Fungal	240-250	α AI1, α AI2	(Marshall and Lauda, 1975; Ho and Whitaker, 1993)
Knottin-like	Amaranth	Insect	32	AAI	(Chagolla-Lopez et al., 1994)
Kunitz-like	Wheat, Barley, Rice, maize, cowpea	Insect, Plant	176-181	BASI, RASI, WASI	(Mundy et al., 1983; 1984; Swensson et al., 1986; Ohtsubo and Richardson, 1992; Iulek et al., 2000; Alves et al., 2009)
γ - Purothionin	Sorghum	Insect, Mammalian	47-48	SI α 1, SI α 2, SI α 3	(Bloch Jr and Richardson, 1994)
Thaumatococcal-like	Maize	Insect	173-235	Zeamatin	(Schmioler-O'Rourke and Richardson, 2001; Franco et al., 2002)
CM-proteins	Wheat, Barley, Rye, ragi	Insect, Mammalian, Bacteria	124-160	0.19,0.28,0.53, RATI (RBI), RP25, WRP26, BMAI-1	(Campos and Richardson, 1983; Mundy et al., 1984; Franco et al., 2000, 2002; Swensson et al., 2004)

Table 2. Classification of plant α -amylase inhibitors based on structural similarity (Richardson, 1990).

3.1.1 Lectin-like inhibitors

There have been particular attentions on lectin-like inhibitors and they are toxic against several insect pests (Ishimoto and Kitamura, 1989; Huesing et al., 1991a; Ishimoto and Chrispeels, 1996; Grossi-de-Sa et al., 1997, Kluh et al., 2005; Karbache et al., 2011). α AI-1 and α AI-2, Two lectin-like inhibitors, were identified in common white, red and black kidney beans (Ishimoto and Chrispeels, 1996;). They show different specificity against α -amylases because of the mutation in their primary structure (Grossi de Sa et al., 1997). α AI-1 inhibits mammalian α -amylases and several insect amylases, but it is not active against Mexican bean weevil (*Zabrotes subfasciatus*). On the other hand, α AI-2 does not inhibit the α -amylases recognized by α AI-1 but inhibits the α -amylase of *Z. subfasciatus* (Ishimoto and Chrispeels, 1996; Kluh et al., 2005).

3.1.2 Knottin-like inhibitors

The major α -amylase inhibitor (AAI) present in the seeds of *Amaranthus hypocondriacus*, is a 32-residue-long polypeptide with three disulfide bridges (Chagolla-Lopez et al., 1996). AAI strongly inhibits α -amylase activity of *Tribolium castaneum* and *Prostephanus truncatus*, however, does not inhibit proteases and mammalian α -amylases. AAI is the smallest proteinaceous inhibitor of α -amylases yet described. Its residue conservation patterns and

disulfide connectivity are related to the squash family of proteinase inhibitors, to the cellulose binding domain of cellobiohydrolase, and to omega-conotoxin, i.e. knottins. The three-dimensional model of AAI contains three antiparallel β strands and it is extremely rich in disulfides (Carugo et al., 2001).

3.1.3 Kunitz-type

Kunitz-like α -amylase inhibitors commonly found in cereals such as barley, wheat and rice (Micheelsen et al., 2008; Nielsen et al., 2004). Recently, they have also reported from legums, e.g. Cowpea (*Vigna unguiculata*) (Alves et al., 2009). Kunitz-like α -amylase inhibitors from Cowpea were active against both insect and mammals α -amylase with different intensity (Alves et al., 2009). α -Amylase/subtilisin inhibitors (BASI) are the most studied inhibitors of the Kunitz-like trypsin inhibitor family (Melo et al., 2002), that have bifunctional action i.e. as a plant defense and also as a regulator of endogenous α -amylase action (Micheelsen et al., 2008; Nielsen et al., 2004). The structure of BASI consists of two disulfide bonds and a 12-stranded β -barrel structure which belongs to the β -trefoil fold family. The interaction of Kunitz-like α -amylase inhibitors with the barley α -amylase 2 (AMY2) revealed a new kind of binding mechanisms of proteinaceous α -amylase inhibitors since calcium ions modulate the interaction (Melo et al., 2002).

3.1.4 γ - Purothionin type

The members of this family contain inhibitors with 47 – 48 amino acid residues that show strongly inhibition activity against insect α -amylases (Bloch Jr and Richardson, 1991). SI α -1, SI α -2 and SI α -3 are three isoinhibitors isolated from *Sorghum bicolor* and showed inhibitory activity against digestive α -amylases of cockroach and locust, poorly inhibited *A. oryzae* α -amylases and human saliva. These inhibitors did not show inhibitory activity on the α -amylases from porcine pancreas, barley and *Bacillus* sp. (Bloch Jr and Richardson, 1991). The three isoforms contain eight cysteine residues forming four disulfide bonds (Nitti et al., 1995).

3.1.5 CM- proteins

CM (chloroform-methanol)-proteins are a large protein family from cereal seeds containing 120 –160 amino acid residues and five disulfide bonds (Campos and Richardson, 1983; Halford et al., 1988). Cereal-type is also refers to these inhibitors since they are present in cereals. CM-proteins show a typical double-headed α -amylase/trypsin domain (Campos and Richardson, 1983). This feature make it possible that they show inhibitory activity against α -amylases (Barber et al., 1986a) and trypsin-like enzymes (Barber et al., 1986b; De Leo et al., 2002) separately or show α -amylases/ trypsin-like inhibitory activity at the same time (Garcia- Maroto et al., 1991). The CM protein family includes lipid transfer proteins (Lerche and Poulsen, 1998; Svensson et al., 1986) and proteins related to cold tolerance (Hinch, 2002). The α -amylase inhibitor 0.19, one of the most studied inhibitor of this family, has a broad specificity and inhibits α -amylases from insects, birds and mammal (Titarenko et al., 2000; Franco et al., 2000; Franco et al., 2002; Oneda et al., 2004). It has 124 amino-acid residues and acting as a homodimer (Oda et al., 1997; Franco et al., 2000). The X-ray crystallographic analysis of 0.19 AI demonstrated that each subunit is composed of four major α -helices, one one-turn helix, and two short antiparallel β -strands. The subunits in a

dimer are related each other by non-crystallographic 2-fold axis, and the interface is mainly composed of hydrophobic residues (Oda et al., 1997).

3.1.6 Thaumatin-like

This family contains proteins with molecular weight about 22 kDa, which are homologous with the intensely sweet protein thaumatin from fruits of *Thaumatococcus daniellii* Benth, thus they are called thaumatin-like (Cornelissen et al., 1986; Vigers., 1991; Hejgaard et al., 1991). Although thaumatin-like proteins is a homologue of the sweet protein thaumatin and exhibit α -amylase inhibitory activity, however, thaumatin and other related proteins do not show inhibitory activity against α -amylases (Franco et al., 2002; Svensson et al., 2004 and references therein). Zeamatin from maize is the best-characterized member of this family which inhibits insect but not mammalian α -amylases. Zeamatin has 13 β strands, 11 of which form a β sandwich at the core of protein (Batalia et al., 1996). Zeamatin has been applied as antifungal drugs because it binds to β -1,3-glucan and permeabilizes fungal cells resulting in cell death (Roberts and Selitrennikoff, 1990; Franco et al., 2000).

4. Insect digestive proteinases

Proteinases, which are also known as endopeptidases, enroll an important function in protein digestion. These enzymes begin the protein digestion process by breaking internal bonds in proteins. The amino acid residues vary along the peptide chain, therefore, different kind of proteinases are necessary to hydrolyze them. Based on active site group and their correspond mechanism, digestive proteinases can be classified as serine, cysteine, and aspartic proteinases (Terra and Ferreira, 2012). Serine, cysteine are the most widespread proteinases in insect digestive system.

Serine proteinases (EC 3.4.21) have the active site composed of serine, histidine, and aspartic acid residues (also called catalytic triad). Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and elastase (EC 3.4.21.36) are the major digestive enzymes of this family that usually work at alkalin pH. These enzymes differ in structural features that are associated with their different substrate specificities. Trypsin are endoproteases that attack proteins at residues of arginine and lysine. Generally, insect trypsins have molecular masses in the range 20–35 kDa, pI values 4–5, and pH optima 8–10 (Terra and Ferreira, 2012). Trypsin occurs in the majority of insects, with the remarkable exception of some hemipteran species and some taxa belonging to the series Cucujiformia of Coleoptera like Curculionidae (Terra and Ferreira, 1994). Nevertheless, some heteropteran Hemiptera have trypsin in the salivary glands (Zeng et al., 2002). Chymotrypsin enzymes attack proteins at aromatic residues (e.g., tryptophan). Insect chymotrypsins usually have molecular masses of 20–30 kDa and pH optima of 8–11 (Terra and Ferreira, 1994). Similar to trypsin, chymotrypsin is also distributed in the majority of insects (Terra and Ferreira, 1994), including those purified from Lepidoptera (Peterson et al., 1995; Volpicella et al., 2006), Diptera (de Almeida et al., 2003; Ramalho-Ortigão et al., 2003), Hemiptera (Colebatch et al., 2002), Hymenoptera (Whitworth et al., 1998), Siphonaptera (Gaines et al., 1999) and Coleoptera (Oliveira-Neto et al., 2004; Elpidina et al., 2005).

Cysteine proteinases occur in the digestive system of insects (Rawlings and Barrett, 1993). These enzymes are also found in other tissue of insects, indicating that they are associated

with other functions in insect (Matsumoto et al., 1997). Cysteine proteinases have their optimum activity in the alkaline range (Bode and Huber, 1992; Oliveira et al., 2003). It has been revealed that cathepsin L-like enzymes are the only quantitatively important member of cysteine proteinases presented in midgut of insects. Digestive cathepsin L-like enzymes have been purified from *Diabrotica virgifera* (Coleoptera: Cucujiformia) (Koiwa et al., 2000), *Acyrtosiphon pisum* (Hemiptera: Sternorrhyncha) (Cristofolletti et al., 2003), *T. molitor* (Coleoptera: Cucujiformia) (Cristofolletti et al., 2005), *Sphenophorus levis* (Coleoptera: Curculionidae) (Soares-Costa et al., 2011; Fonseca et al., 2012), and *Triatoma brasiliensis* (Reduviidae, Triatominae) (Waniek et al., 2012).

5. Proteinase inhibitors from plants

PIs are a natural plant defensive mechanism against insect herbivores which were viewed as promising compounds for developing insect resistance transgenic crops that over-express PIs (Gatehouse, 2011). PIs have found in animals, plants (particularly legumes and cereals), and microorganisms. Most storage organs such as seeds and tubers contain 1-10% of their total proteins as PIs with different biochemical and structural properties inhibiting different types of proteases (Volpicella et al., 2011). PIs play an important role in different physiological functions of plants including as storage proteins, and regulators of endogenous proteolytic activity (Ryan, 1990), modulators of apoptotic processes or programmed cell death (Solomon et al., 1999), and defense components associated with the resistance of plants against insects and pathogens (Lu et al., 1998; Pernas et al., 1999). Green and Ryan (1972) pioneer works revealed the roles of PIs in the plant-insect interaction. They showed induction of plant PIs in response to attack of insects and pathogen and named this induction as “defense-response” of the plant against the pests. Production of PIs that inhibit digestive herbivore gut proteases inspired the field of plant-insect interactions and became an outlandish example of induced plant defenses. Since then, several PIs of insect proteinases have been identified and characterized (Garcia-Olmedo et al. 1987; Lawrence and Koundal 2002). Despite insects that feed on sap or seeds, most phytophagous insects are nutritionally limited by protein digestion. Since plant tissues are nitrogen deficient compared to insect composition, and the main source of nitrogen available to the insect is protein (Gatehouse, 2011). Therefore, their proteinases have an important role in digestion of proteins and maintaining of needed nitrogen. Inactivation of digestive enzymes by PIs results in blocking of gut proteinases that leads to poor nutrient utilization, retarded development, and death because of starvation (Jongsma and Bolter 1997; Gatehouse and Gatehouse, 1999).

There have been considerable number of reviews on plant PIs describing their classification (Turra et al., 2011; Volpicella et al., 2011), biochemical and structural properties (Antao and Malcata, 2005; Bateman and James, 2011; Oliva et al., 2011), their role in plant physiology (Schaller, 2004; Salas et al., 2008; Roberts and Hejgaard, 2008), insect-plant co-evolution (Jongsma and Beekwilder, 2011), and their application in different areas including pest control (Lawrence and Koundal, 2002; Gatehouse, 2011), nutritional (Clemente et al., 2011) as well as pharmaceutical (Gomes et al., 2011) applications.

5.1 Plant proteinase inhibitors classes

PIs are classified based on the type of enzyme they inhibit: Serine protease inhibitors, cysteine protease inhibitors, aspartic protease inhibitors, or metallocarboxy-protease

inhibitors (Ryan, 1990; Mosolov, 1998; Bode and Huber, 2000). Plant serine proteinase inhibitors further sub-classified to a number of subfamilies based on their amino acid sequences and structural properties known as Kunitz type, Bowman-Birk type, Potato I type, and Potato II type inhibitors (Bode and Huber, 1992). The families of PIs could not, however, be grouped on the basis of the catalytic type of enzymes inhibited, since a number of families contain cross-class inhibitors. Despite cysteine and metallocarboxy inhibitor families, all other reported families of PIs contain inhibitors of serine proteases. (Volpicella et al., 2002). The proteins in Kunitz-like family, for instance, generally inhibit serine proteinases, besides they also include inhibitors of cysteine and aspartate proteases (Heibges et al., 2003). There are some exceptions, however, that PIs families may have not inhibitors of serine proteases such as aspartic protease inhibitors in Kunitz and cysteine families and also potato cysteine protease inhibitors that belongs to Kunitz family (Volpicella et al., 2002).

6. Transgenic plants expressing digestive enzyme inhibitors

It seems obvious that the prospective amylase and proteinase inhibitors can function as a biotechnological tool for the discovery of novel bioinsecticides or in the construction of transgenic plants with enhanced resistance toward pests and pathogens.

Since Johnson et al. (1989) expressed proteinase inhibitors in transgenic tobacco providing enhanced resistance against *Manduca sexta* larvae, hundreds of reports have been produced in this specific issue. As previously described, proteinase inhibitors could act on the digestive enzymes of insect herbivores reducing food digestibility. Attempts to achieve this defense mechanism in plants, genetic engineering have used over-expression of both exogenous and endogenous proteinase inhibitors (Gatehouse, 2011).

Among several targets, Lepidopteran has been clearly focused, since they are important groups of crop insect-pests in the world. Until now the only commercially accessible transgenes for control of these insect pests encode Cry *Bacillus thuringiensis* (Bt) toxins and the Vip3Aa20 toxin (United States Environmental Protection Agency, 2009). Several trials have been performed by using proteinase inhibitors. For example the mustard trypsin inhibitor (MTI-2) was expressed at different levels in transgenic tobacco, Arabidopsis and oilseed rape lines. The three plants were challenged against different lepidopteran insect-pests, including *Plutella xylostella* (L.), which was extremely sensible to MTI-2 ingestion being completely exterminated (de Leo et al., 2001). Furthermore MTI-2 was also expressed at different levels in transgenic tobacco lines and was further appraised by feeding of the lepidopteran larvae, *Spodoptera littoralis* (de Leo and Galerani et al., 2002). A surprising result was obtained. *S. littoralis* larvae feed on transgenic tobacco expressing MTI-2 were unaffected. However, significant reduction on fertility was obtained suggesting that multiple effects could be obtained with a single proteinase inhibitor. In this view, several research groups have produced and evaluated transgenic plants synthesizing proteinase inhibitors and attacked by Lepidoptera pests. Among inhibitors expressed in transgenic plants were NaPI, the *Nicotiana glauca* proteinase inhibitor and also the multidomain potato type II inhibitor that is produced at enhanced levels in the female reproductive organs of *N. glauca* (Dunse et al., 2010). The individual inhibitory domains of NaPI target trypsin and chymotrypsin, from digestive tract of lepidopteran larval pests. While feeding on NaPI, dramatically reduced the *Helicoverpa punctigera* growth, surviving larvae exhibited high

levels of chymotrypsin resistant to inhibition by NaPI. In order to solve this problem, NaPI was expressed in synergism with *Solanum tuberosum* potato type I inhibitor (StPin1A), which strongly inhibited NaPI-resistant chymotrypsins. The mutual inhibitory effect of NaPI and StPin1A on *H. armigera* larval growth was observed both in laboratory conditions as well as in field trials of transgenic plants.

Improved crop protection achieved using mixtures of inhibitors in which one class of proteinase inhibitor is utilized to contest the genetic ability of an insect to adapt to a additional class of proteinase inhibitor. Furthermore, amylase inhibitors have also been utilized as defense factors against insects in genetic modified plants. Several amylase inhibitors have been expressed in different plants. However the expression of α -amylase inhibitors (α -AI) from scarlet runner bean (*Phaseolus coccineus*) and common bean (*Phaseolus vulgaris*) has been extremely protective in genetic modified plants, showing enhanced shelter against pea weevils (Shade et al., 1994; Schroeder et al., 1995), adzuki bean (Ishimoto et al., 1996), chickpea (Sarmah et al., 2004; Ignacimuthu et al., 2006, Campbell et al., 2011) and cowpea (Solleti et al., 2008). Furthermore, transgenic pea showed enhanced defense against the pea weevil *Bruchus pisorum* was shown under field conditions (Morton et al., 2000). All these trials associated the α -AI expression with the seed-specific promoter of phytohemagglutinin from *P. vulgaris*.

Moreover other crops, in addition to legumes, were also transformed with amylase inhibitors. The Rubiaceae *Coffea arabica* was also engineered with α -AI1 under control of phytohemagglutinin promoter (Barbosa et al., 2010). The presence of this gene was observed by PCR and Southern blotting in six regenerated transgenic T1 coffee plants. Immunoblotting and ELISA experiments using antibodies against α -AI1 revealed the presence of this inhibitor at a concentration of 0.29 % in seed extracts. The presence of this inhibitor was able to cause a clear inhibitory activity on digestive enzymes of *Hypotenemus hampei* suggesting a possible protective effect.

Also, an α -amylase inhibitor from cereal-family (BIII) from rye (*Secale cereale*) seeds was also cloned and expressed initially in *E. coli* showing clear activity toward α -amylases of larvae of the coleopteran pests *Acanthoscelides obtectus*, *Zabrotess subfasciatus* and *Anthonomus grandis* (Dias et al. 2005). BIII inhibitor was also expressed under control of phytohemagglutinin promoter in tobacco plants (*Nicotiana tabacum*). Besides, the occurrence of BIII-rye gene and further protein expression were confirmed. Immunological analyzes indicated that the recombinant inhibitor was produced in concentration ranging from 0.1% to 0.28% (w: w). Bioassays using transgenic seed flour for artificial diet caused 74% mortality for cotton boll weevil *A. grandis* suggesting that rye inhibitor could be an auspicious biotechnological tool for yield transgenic cotton plants with an improved resistance to weevil (Dias et al., 2010).

7. Summary

While important protection against insect pests has been routinely achieved, the transgenic plants do not show levels of resistance considered commercially possible. As a consequence of selective pressures, insect herbivores have developed various adaptation mechanisms to overcome the defensive effects of plant inhibitors. Common polyphagous crop pests are well adapted to avoid a wide range of different inhibitors, which have only limited effects.

Multiple strategies have been attempted to improve effectiveness of digestive enzyme inhibitors towards insects, including selection for inhibitory activity toward digestive enzymes, mutagenesis for novel inhibitory activity, and engineering multifunctional inhibitors. However, digestive enzyme inhibitors have only been used in genetic modified crops in mishmash with other insecticidal genes. In genetically engineered cotton plants which express Bt toxins, the CpTI gene has been employed as an additional transgene to improve protection against lepidopteran larvae. This gene combination indicates the only commercial disposition of a proteinase inhibitor transgene to date, with Bt/CpTI cotton grown on over 0.5 million hectares in 2005. Until now, no amylase inhibitor was commercially utilized. Future predictions for using digestive enzyme inhibitor genes to boost insect resistance in transgenic crops will require reconsideration of their mechanisms of action, particularly in disturbing processes other than ingestion.

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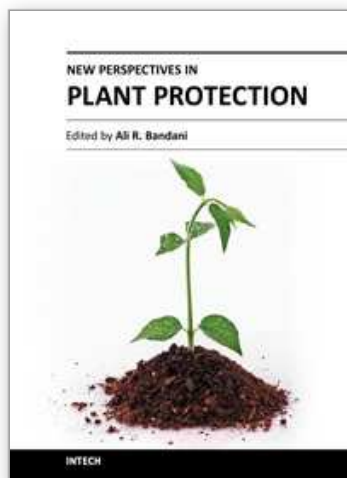
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Crop losses by pests (insects, diseases and weeds) are as old as plant themselves but as agriculture are intensified and cropping patterns including the cultivation of high yielding varieties and hybrids are changing over time the impact of the pests becoming increasingly important. Approximately less than 1000 insect species (roughly 600-800 species), 1500 -2000 plant species, numerous fungal, bacterial and nematode species as well as viruses are considered serious pests in agriculture. If these pests were not properly controlled, crop yields and their quality would drop, considerably. In addition production costs as well as food and fiber prices are increased. The current book is going to put Plant Protection approaches in perspective.

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