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REVIEW

Insect digestive enzymes as a target for pest control

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Abstract

The continual need to increase food production necessitates the development and application of novel biotechnologies to enable the provision of improved crop varieties in a timely and cost-effective way. Plants and herbivores have been co-evolving for thousands of years, and as a result, plants have defence mechanisms that offer protection against many herbivores/predators. Plant proteinase inhibitors (PIs), which play a potent defensive role against predators and pathogens, are natural, defense-related proteins often present in seeds and induced in certain plant tissues by herbivory or wounding. This review describes the main classes of proteinase inhibitors and proteinases, their distribution and localization, general properties, and their main functions. Possible applications utilities for the PI and proteolytic enzymes in plant biotechnology have been reviewed.

Key Words: proteinase inhibitor; herbivory; plant; biotechnology

Introduction

Losses of agricultural production due to pests and diseases have been estimated at 37 % in Europe and worldwide. Most damage is caused by arthropods and the methods available today for protecting plant crops against insect predation are heavily dependent on environmentally-aggressive chemicals and that have been estimated to reduce losses by only about 7 % (Oerke *et al*., 1994). This fact justifies the necessity for the research and development of alternative approaches to this problem (Carlini and Fatima, 2002).

Plant defenses against insect herbivores are mediated, in part, by enzymes that impair digestive processes in the insect gut. Little is known about the evolutionary origins of these enzymes, their distribution in the plant kingdom, or the mechanisms by which they act in the protease-rich environment of the animal digestive tract (Chen *et al*., 2007).The transgenic expression of insecticidal proteins such as α-amylase and protease inhibitors is also being evaluated as a potential protective strategy against insects (Schuler *et al*., 1998).

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Digestive proteinases of insects

The proteinases are a major group of hydrolytic enzymes in insects and are involved in digestive processes, proenzyme activation, liberation of physiologically active peptides, complement activation, and inflammation processes amongst others (Neurath, 1984). The proteinases are classified according to their mechanism of catalysis: (1) serine proteinases; (2) cysteine proteinases; (3) aspartic proteinases, and (4) metalloproteinases (Bode and Huber, 1992).

For an efficient management of pest control through proteinase inhibitor transgenes, it is imperative to know the type of enzymes present in the gut of insects and pests. The two major proteinase classes in the digestive systems of phytophagous insects are the serine and cysteine proteinases (Haq *et al*., 2004).

Murdock *et al.* (1987) carried out an elaborate study of the midgut enzymes of various pests belonging to Coleoptera, while Srinivasan *et al.* (2008) have reported on the midgut enzymes of various pests belonging to Lepidoptera. Serine proteases are known to dominate the larval gut environment and contribute to about 95 % of the

total digestive activity in Lepidoptera, whereas the Coleopteran species have a wider range of dominant gut proteinases.

Cysteine proteinases

Cysteine proteinases, endopeptidyl hydrolases with a cysteine residue in their active center are usually identified based on the effect of their active site inhibitors (iodoacetate, iodoacetamide and E-64) and activation of the enzymes by thiol compounds (Grudkowska and Zagdańska, 2004).

In insects, the cysteine proteinases are utilized in the digestive processes (Rawlings and Barrett, 1993), but are found in several other tissues, indicating that they may also play other roles (Matsumoto *et al.*,1997). Studies on the pH dependence of cysteine proteinase activity in the crude extract of insect larvae have indicated that this activity was generally in the alkaline range (Bode and Huber, 1992; Oliveira *et al.*, 2003).

The papain family contains peptidases with a wide variety of activities, including endopeptidases with broad specificity (such as papain), endopeptidases with very narrow specificity (such as glycyl endopeptidases), aminopeptidases, dipeptidyl-peptidase, and peptidases with both endopeptidase and exopeptidase activities (such as cathepsins B and H). There are also family members that show no catalytic activity (Dubey *et al*., 2007).

The three-dimensional structure of papain, a representative member of papain-like cysteine proteases (Kamphuis *et al*., 1984), has been elucidated (Fig. 1), as well as other members. All papain-like cysteine proteases share similar sequences (Berti and Storer, 1995) and have similar 3-dimensional structures. The structural data provides strong evidence that these proteinases all arose from a common ancestor (Dubey *et al*., 2007). Proteinaceous inhibitors of cysteine proteinases are subdivided into three families (stefin, cystatin and kininogen) based on their sequence homology, the presence and position of intrachain disulfide bonds, and the molecular mass of the protein (Turk and Bode, 1991).

Serine proteinases

Serine proteinases are widely distributed in nearly all animals and microorganisms (Joanitti *et al*., 2006). In higher organisms, nearly 2 % of genes code for these enzymes (Barrette-Ng *et al.*, 2003). Being essentially indispensable to the maintenance and survival of their host organism, serine proteases play key roles in many biological processes. Serine proteases are classically categorized by their substrate specificity, notably by whether the residue at P1: trypsin-like (Lys/Arg preferred at P1), chymotrypsin-like (large hydrophobic residues such as Phe/Tyr/Leu at P1), or elastase-like (small hydrophobic residues such as Ala/Val at P1) (revised by Tyndall *et al.*, 2005). Serine proteases are a class of proteolytic enzymes whose central catalytic machinery is composed of three invariant residues, an aspartic acid, a histidine and a uniquely

Fig. 1 Molscript diagram of the final model of papain, in complex with CCPI (cowpea cystatin proteinase inhibitor). The papain portion (below) is shown as a gray Cα trace and the cystatin (above) as a ribbon diagram. Side chains of cystatin residues interacting with the enzyme are drawn in a ball-and-stick representation and labeled. The orientation was chosen to clearly display the role of the inhibitor N-terminus, leading to partial concealment of residue Glu 18 (Aguiar *et al.*, 2006).

reactive serine, the latter giving rise to their name, the “catalytic triad” (Fig. 2).

The Asp-His-Ser triad can be found in at least four different structural contexts (Hedstrom, 2002). These four clans of serine proteases are typified by chymotrypsin, subtilisin, carboxypeptidase Y, and Clp protease. The three serine proteases of the chymotrypsin-like clan that have been studied in greatest detail are chymotrypsin, trypsin, and elastase. All three of these enzymes are similar in configuration, as shown by their X-ray structures (Figs 2, 3). More recently, serine proteases with novel catalytic triads and dyads have been discovered, including Ser-His-Glu, Ser-Lys/His, His-Ser-His, and N-terminal Ser (Hedstrom, 2002).

Proteinase inhibitors

Inhibitor proteins have been found for each of the four mechanistic classes of proteinases and a large number of proteinase inhibitors are directed towards serine- and cysteine proteinases (Barrett *et al.*, 1987; Turk and Bode, 1991). In contrast, only a few of these inhibitors are known for aspartic- and metalo-proteinases (Jouanin *et al*., 1998; Oliveira *et al*., 2003).

Plant proteins that inhibit various types of enzymes from a wide range of organisms have

been extensively studied. Proteinase inhibitors (PIs) comprise one of the most abundant classes of proteins in plants. Most storage organs such as seeds and tubers contain 1 to 10 % of their total proteins as PIs, which inhibit different types of enzymes (Ryan *et al*., 1981). Inhibitors bind tightly to the enzyme's active site in a substrate-like manner, resulting in a stable complex unlike that of the weak complexes between enzyme-substrate and enzyme-product, which dissociate in a short spantime (Oliva *et al*., 2010). The function of the inhibitors is to control proteolysis within cells, organelles or fluids when limited proteolysis is important for the biochemical or physiological process.

A large number of transgenic plants have been developed with PIs that confer resistance to different families of insects. Serine proteinases inhibitors have been the subject of more research than any other class of proteinase inhibitors and are effective against the serine proteinases in the gut of many insect families, particularly Lepidoptera. The role of PIs against herbivory was hypothesized due to the abundance of these proteins and the lack of activity against endogenous proteins. Extensive studies have shown that PIs are induced as components of many defense cascades under various stress-prone conditions, such as insect attack and mechanical wounding (Ryan, 1990)

Cystatin

The name cystatin was first used by Barrett (1981) to describe an inhibitor that had been discovered and partially characterized from chicken egg-white of papain, ficin and other related cysteine (Sen and Whitaker, 1973). When other protein inhibitors of cysteine proteinases were characterized and their amino acid sequences determined, it became apparent that these are related to chicken cystatin and, thus, are members of the cystatin superfamily (Barrett *et al*., 1986).

Cystatins, similarly to other competitive protease inhibitors, form a tight complex with the active site of target proteases to cause inhibition and interfere with dietary protein digestive functions in herbivorous organisms (Arai *et al*., 2002).

Phytocystatins

Plant cystatins or phytocystatins are the second most studied class of inhibitors and have been identified and characterized from several plants, including cowpea, potato, cabbage, ragweed, carrot, papaya, apple fruit, avocado, chestnut, and Job's tears, among others. Cystatins have also been isolated from seeds of a wide range of crop plants. These crop plants include those of the sunflower, rice, wheat, maize, soybean, sugarcane, etc. (Revised by Haq *et al*., 2004).

The phytocystatins (5 - 87 kDa) present characteristics found in the cystatin subfamilies I and II (Arai *et al*., 2002). Most phytocystatins have a molecular mass in the 12 - 16 kDa range and are devoid of disulphide bonds and of putative glycosylation sites. Several cysteine proteinase inhibitors have been identified and their primary and

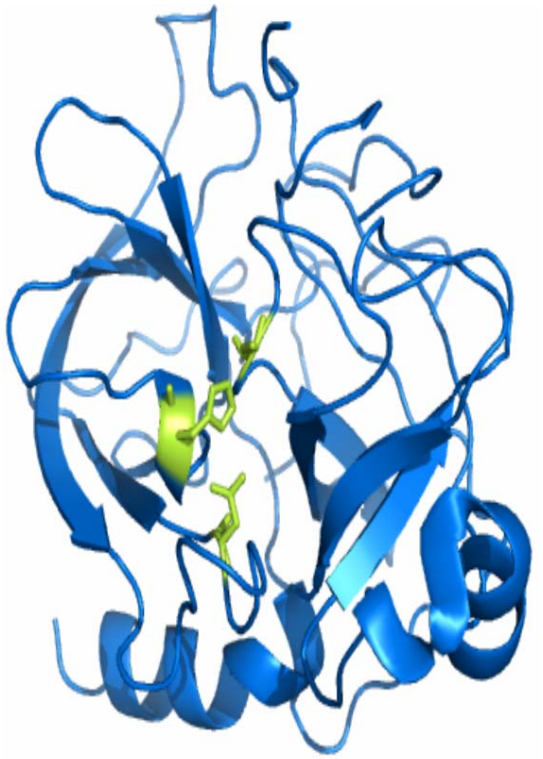

Fig. 2 Crystal structure of bovine chymotrypsin. The catalytic residues are shown as yellow sticks. Rendered from PDB 1CBW.

tertiary structures were determined (Fig. 1) (Aguiar *et al.*, 2006). Some of these show homology with serine proteinase inhibitors, such as the potato tuber cysteine proteinase inhibitor that belongs to the Kunitz-type trypsin inhibitor family, and do not contain the conserved region (Gln-X-Val-Y-Gly) that characterizes the cystatin superfamily (Carlini and Grossi-de-Sá, 2002**)**.

Scientific articles have been published reporting on the role of phytocystatins in the control of Cys protease activities in plants, or their potential as potent inhibitors of Cys proteases in biological systems of practical interest. Plant cystatins are now known to be involved in a large variety of physiological processes, ranging from the control of endogenous proteolysis in reproductive and vegetative organs to the inhibition of digestive, extracellular Cys proteases of herbivore arthropods, parasitic nematodes and microbial pathogens (revised by Benchabane *et al*., 2010).

Serine proteinase inhibitors

Protease Inhibitors (PIs), which are ubiquitous in nature, are a group of prime pest-control candidates, with highly proven inhibitory activities against insect pests and the ability to suppress the enzymatic activity of phytopathogenic micro organisms and nematodes. The possible role of PIs in plant protection was investigated as early as 1947 by Mickel and Standish and the first transgenic

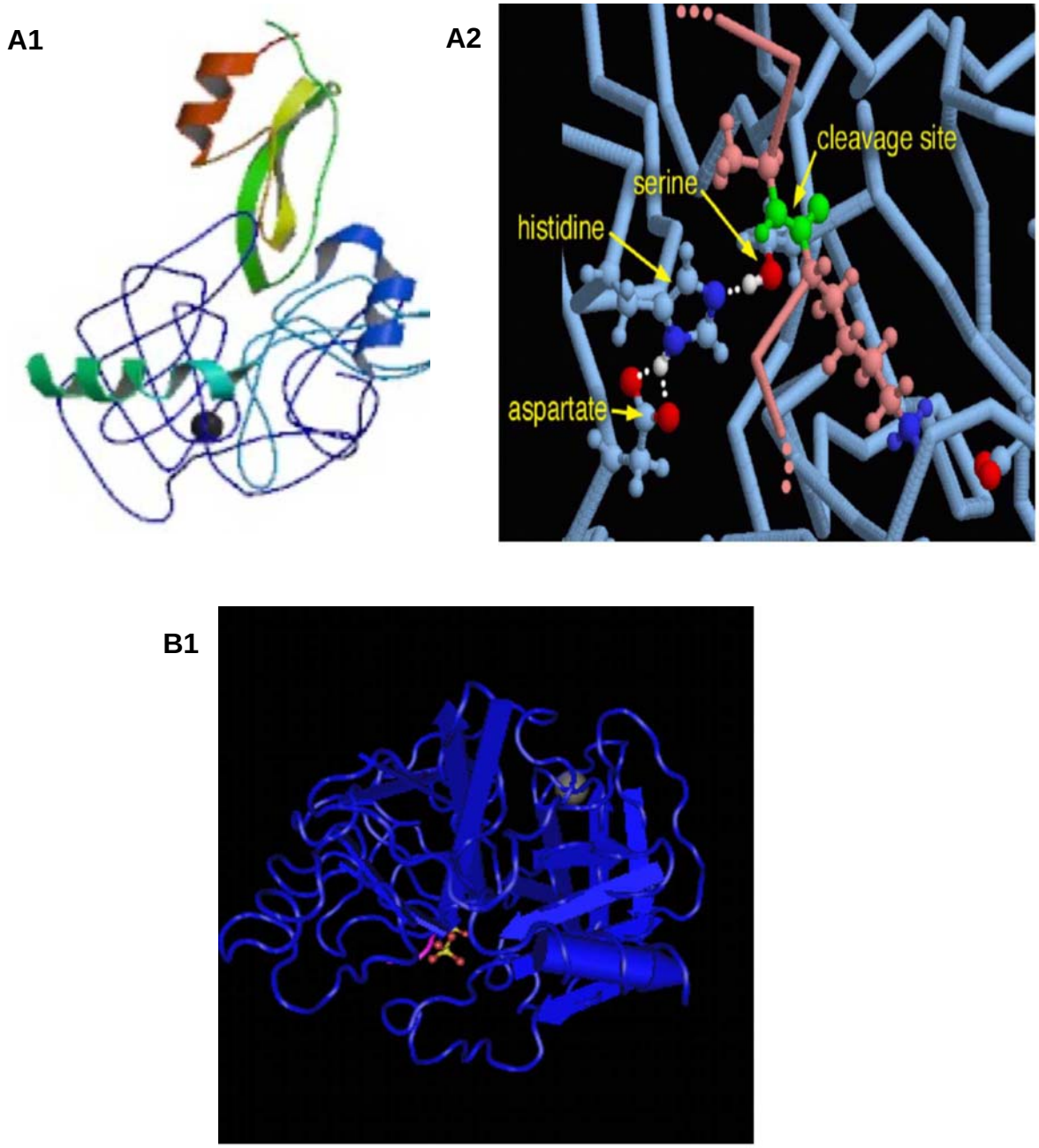

Fig. 3 X-ray crystallographic structure of serine proteinases. (A1) Trypsin structure and (A2) structure catalytic triad in detail (PDB ID: 2PTC); (B) Elastase (PDBI ID: 1GVK)

tobacco plant expressing PIs was first reported in 1987 (Hilder *et al*., 1987).

Plant proteins that inhibit various types of enzymes from a wide range of organisms have been extensively studied. Proteinase inhibitors (PIs) comprise one of the most abundant classes of proteins in plants. Most storage organs such as seeds and tubers contain 1 to 10 % of their total proteins as PIs, which inhibit different types of enzymes (Ryan *et al*., 1981). Inhibitors bind tightly to the enzyme's active site in a substrate-like manner, resulting in a stable complex unlike that of the weak complexes between enzyme-substrate and enzyme-product, which dissociate in a short spantime (Oliva *et al*., 2010). The function of the inhibitors is to control proteolysis within cells, organelles or fluids when limited proteolysis is important for the biochemical or physiological process.

One of the recent developments in the field of plant genetic engineering is the manipulation of plants for disease and insect resistance. In an effort to develop insect-resistant crops, the role of plant-derived PIs was recognized early on. By transferring a single defensive gene from one plant to another either with its own promoter or with constitutive

promoters, genetically modified plants can be readily obtained. A large number of transgenic plants have been developed with PIs that confer resistance to different families of insects. Serine proteinases inhibitors have been the subject of more research than any other class of proteinase inhibitors and are effective against the serine proteinases in the gut of many insect families, particularly Lepidoptera. The role of PIs against herbivory was hypothesized due to the abundance of these proteins and the lack of activity against endogenous proteins. Extensive studies have shown that PIs are induced as components of many defense cascades under various stress-prone conditions, such as insect attack and mechanical wounding (Ryan, 1990)

The size of plant proteinase inhibitor (PI) proteins ranges from 4 to 85 kDa, with a great proportion being small proteins of only 8 - 20 kDa. Their amino acid composition is enriched in cysteine residues that are significant in the formation disulfide bridges and in conferring stability to heat, pH changes, and proteolysis (revised by Chye *et al*., 2006). The serine proteinase inhibitors are found in plants including the Kunitz (soybean trypsin inhibitor) family, the Bowman-Birk (soybean proteinase inhibitor) family, potato I inhibitor family, potato II inhibitor family, barley trypsin inhibitor family, and squash inhibitor family (Norton, 1991). Bowman-Birk type inhibitors are small polypeptides (8 kDa), typically found in legume seeds. They are double-headed, binding simultaneously and independently to two separate proteinase molecules, such as trypsin and chymotrypsin (Bode and Huber, 1992). Plant Kunitz inhibitors are widely distributed in plants and are mainly concentrated in leguminous seeds of the taxonomic subfamilies Mimosoideae, Caesalpinioideae and Papilionoideae. The structural pattern of most plant Kunitz inhibitors is a single polypeptide chain of approximately 20 kDa with two disulfide bonds (Cys39-Cys86 and Cys136-Cys145) and a single reactive site (Oliva *et al.*, 2010). Some plant serine proteinase inhibitors are bifunctional molecules and are able to inhibit trypsins as well as α-amylase (Strobl *et al*., 1995). Recently, two kunitz inhibitors from *Prosopis juliflora* and *Adenanthera pavonina* have been shown to possess potent cysteine-inhibitor activity (Franco *et al*., 2002; Macedo *et al*., 2004).

Developing insect resistant transgenic plants expressing proteinase inhibitors

During the past decade, fundamental changes have taken place in the field of plant molecular biology. Among the large number of new technologies that are available, commercial interest has focused on the ability of plants to integrate and express foreign genes and to produce recombinant proteins in bulk quantities at a relatively low cost (Franken *et al*.,1997). New inhibitors against predatory insects with the potential for use in plant-genetic engineering to develop transgenic resistant plants have been characterized (Ussuf *et al*., 2001).

The main function of plant cysteine protease inhibitors is thought to be for plant defense.The defensive role of plant cystatins may be due to their inhibitory activities towards the digestive enzymes of insects, their larvae and other proteases involved in some vital processes. Several other transgenic plants expressing cysteine protease inhibitors have been shown to be effective against phytophagous insects. (Mosolov and Valueva, 2005; Dubey *et al.*, 2007).

The expression of cystatins in transgenic plants to increase host-plant resistance has only been marginally successful. For example, transgenic potatos expressing rice cystatin inhibited larval growth and exhibited mortality of the Colorado potato beetle (Lecardonnel *et al.*, 1999). However, growth compensation and faster development of the same species feeding on potato foliage expressing rice cystatin has also been observed (Cloutier *et al*., 2000). In two varieties of transgenic poplar, expressing cysteine proteinase inhibitors from rice (Leple *et al*., 1995) and *Arabidopsis* (Delledonne *et al*., 2001), substantial levels of resistance to two chrysomelid beetle species were achieved. In an artificial diet, soyacystatin N, a soybean cysteine proteinase inhibitor, inhibited both western corn rootworm gut proteolysis and larval growth (Kiowa *et al*., 2000). Apparently, one or more cathepsin L-like cysteine proteinases of the papain superfamily, present in the rootworm gut, are the targets of this inhibitor (revised by Fabrick *et al*., 2002*).*

The presence of inhibitory domains in serine proteinase inhibitor prompted us to question their physiological functions. It was thought that many plant serine proteinase inhibitor proteins do not have endogenous functions against plant proteases, but show specificities for animal or microbial enzymes. As such, these inhibitor proteins could be applied to combat invasion by pests or pathogens due to their action on foreign proteolytic enzymes. Its actions on insect gut proteases were nonetheless experimentally demonstrated using artificial diets and *in vitro* inhibition assays on insect gut proteases (revised by Chye *et al*., 2006; Macedo *et al*., 2004; Ramos *et al*., 2008).

The CpTI gene isolated from the cowpea plant (*Vigna unguiculata*) has been extensively used in the generation of insect resistant plants. This is the first plant-originated insect resistance gene to be successfully transferred into other plants species (Hilder *et al.*, 1987). CpTI is a member of the Bowman-Birk superfamily of protease inhibitors and possesses the insecticidal properties against the insect groups of Lepidoptera, Coleoptera and Orthoptera (Gatehouse *et al.*, 1997). Cowpea trypsin inhibitor gene CpTI has also been introduced into *Brassica oleracea* var. *capitata* cultivars Yingchun and Jingfeng (Fang et al. 1997). The transformed plants showed resistance to *Pieris rapae* in laboratory tests. Transgenic tobacco expressing high levels of Kunitz type of trypsin inhibitor from soybean demonstrated resistance *Helicoverpa virescens* (Sharma *et al.*, 2000).

PIs of the potato inhibitor I and II family (PIN1 and PIN2) are the best characterized plant serine PIs in terms of their molecular properties (Sin and Chye, 2004). The heterologous expression of PIN1 and PIN2 proteins confers insect resistance in transgenic plants. PIN1 and PIN2 inhibitor target the digestive serine proteinases trypsin and

chymotrypsin, the major enzymes contributing to protein digestion in the gut of lepidopteran larvae. Jonhson *et al.* (1989) expressed PIN 1 and II inhibitors in transgenic tobacco plants. The authors shown that the growth of *Manduca sexta* larvae (tobacco hornworms) feeding on leaves of transgenic plants containing inhibitor II was significantly retarded, compared to growth of larvae fed untransformed leaves. However, the presence of tomato inhibitor I protein, a potent inhibitor of chymotrypsin but a weak inhibitor of trypsin, in transgenic tobacco leaves had little effect on the growth of the larvae.

Currently, the genes of more than 14 proteins, proteinase inhibitors, are expressed in various cultured plants. The majority of transgenic plants containing proteinase inhibitor genes are characterized by increased resistance to insects and some other pests. Apparently, the most promising are plants containing the genes of proteinase inhibitor in combination with genes of other proteins. It can be assumed that in these cases proteinase inhibitors not only act by themselves, but also protect other recombinant proteins from the destructive action of plant proteinases (Valueva and Mosolov, 2004).

Adaptive strategies from insects to proteinase inhibitors

When ingested protease inhibitors (PI) block protease activity and increase insect mortality by restricting the availability of essential amino acids. Mechanisms of insect resistance to PIs include the upregulation of enzymes that degrade the PIs (Yang *et al*., 2009), the induction of enzymes that resist inactivation by PIs (Broadway, 1996), and overproduction of enzymes to maintain normal levels of gut proteolysis (Brioschi *et al*., 2007).

Some insects exhibit an amazing flexibility in adapting to various host plants by altering the specificities of their gut proteases in response to qualitative changes in dietary protein content and when the existing proteases are ineffective and/or inefficient for digestion (Gatehouse *et al.*, 1997). Studies on insect responses to the dietary incorporation of plant-derived PIs have indicated a biphasic response characterized by an initial upregulation of all digestive protease specificities, which precedes a simultaneous downregulation of PI-sensitive proteases and upregulation of PI-insensitive proteases (Bown *et al.*, 2004). A similar response can be expected with a change in host plant. Although it is clear that insects are able to express a variety of proteinases in response to PI exposure, the mechanism of enzyme induction is unknown. Brito *et al*. (2001) found that *Heliothis virescens* larvae vary their complement of trypsin activities when fed control or inhibitor-containing diet. Data indicated that newly-synthesized trypsins have altered substrate specificities, probably reflecting different interactions with substrates and plant inhibitors. Volpicella *et al*. (2003) showed that *Helicoverpa zea* larvae express two different trypsins depending on whether larvae were fed control or inhibitor-containing diets. These enzymes differ in some residues predicted to be involved with inhibitor interactions (Lopes *et al.*, 2004). Other strategies involve the overexpression of proteases from alternative functional classes following inhibitor ingestion (Rivard *et al*., 2004), the degradation of inhibitor with non-target insensitive proteases (Zhu-Salzman *et al*., 2003), and a reallocation of cellular resources towards inhibitor-induced compensatory processes (Liu *et al*., 2004). It is now well recognized that protease/ inhibitor interactions in plant-insect systems are the result of a long co-evolutionary process triggering the continuous diversification of (insect) proteolytic and (plant) protease inhibitory functions (Kiggundu *et al*., 2006; Benchabane *et al*., 2010)

Mechanism of action in insect guts

The mode of action of PIs at the tissue level in insect guts is extremely selective and different types of PIs have a different mechanism of action. The activity of PIs is due to their capacity to form stable complexes with target proteases, blocking, altering or preventing access to the enzyme active site. PIs with activity against serine proteases, the most widespread in nature, act as a potential substrate for proteases. Residues forming the scissile peptide bond are indicated as P1-P1' and are generally located on an external loop of the protein, interacting with proteases. The P1 residue determines the specific type of serine protease inhibited. Other residues around the reactive site also play a role in determining the strength of the PI-enzyme interaction (Fan and Guo-Jiang, 1997).

The possible role of PIs in plant protection was envisaged as early on as 1947 when Mickel and Standish observed that the larvae of certain insects were unable to develop normally on soybean products (Haq *et al.*, 2004). Reese (1983) had proposed a simple hypothesis that growth rates were reduced due to reduced rates of proteolysis, which was later dismissed when Broadway and Duffey examined the physiological effects of PIs in the gut protease activity in insects. These authors suggested that a feedback mechanism led to the hyperproduction of proteinases to compensate for the loss of activity, which in turn led to the depletion of essential amino acids and finally resulted in retarded growth rates. In a study conducted by Marchetti *et al*. (2000), it was observed that larvae fed on transgenic plants expressing a Kunitz inhibitor gradually lost their turgor and became shrunken; hence it appears that food avoidance also has a dramatic effect on the water balance of the feeding larvae (revised by Lawrence *et al*., 2002**)**.

Ramos *et al.* (2009) suggested that the toxic effect of the protease inhibitors induces the insect to eliminate its digestive enzymes in feces, complicating its digestion. In contrast, some insects, such as *Spodoptera littoralis* (Lepidopteran), can overcome the deleterious effects of protease inhibitors by synthesizing different proteases that are insensitive to particular inhibitors (Paulillo *et al*., 2000; Brito *et al*., 2001; De Leo *et al*., 2001; Volpicella *et al*., 2003). Hence, the exact mechanism of action of PIs, at the tissue level of insects, is not well described (Carlini *et al*., 2002; Amirhusin *et al*., 2007).

Future trends

The aim of this literature review was to highlight the ability of some proteins, including PIs, as resistant factors against some important insect pests to reduce the massive use of chemical compounds. These proteins have demonstrated direct insecticidal activity on a wide range of insect pests and have the potential for expression in transgenic crops, conferring insect resistance to plants.

A considerable amount of transgenic plants expressing the genes for serine and cysteine proteinases have been obtained over two decades of research (Valueva *et al*., 2004). Since the discovery that economically important insect pests, namely Lepidoptera, Diptera and Coleoptera, use serine and cysteine proteinases in their digestive system to degrade proteins in ingested food, efforts have been directed at defining genes encoding PIs that are active against these mechanistic classes of proteases for developing transgenic plants (Habib and Fazili, 2007). In a number of cases, the degree of plant protection (assessed by the level of their damage or the effects on the insects) was as high as 50%. However, this value is still lower than those obtained for plants harboring the genes of Bt toxins (95% or higher) (Gatehouse, 2008). The main reason consists of the rapid adaptation of the digestive tract of phytophagous insects to the effects of the inhibitors, which occur due to the genetic diversity of proteolytic enzymes. Further refinement of the method requires new, more efficient proteinase inhibitors to be identified (or those already known or modified, including by constructing hybrid proteins) (revised by Mosolov and Valueva, 2008).

The insect midgut reportedly contains centimes different proteases (Bown *et al*., 1997). These are differentially regulated and cannot all be inhibited by a plant's PIs (Broadway, 1996). With the development of transgenic, insect- and pest-resistant crop varieties, the proteinase inhibitor genes can make a promising contribution towards maximizing yields and minimizing losses due to insects and pests. We can anticipate a number of promising possibilities for pest control through insecticidal genes. All need to be explored and prudently tapped for their implementation in integrated pest management programs (revised by Fan and Wu, 2005)

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