Overviews of biochemical, genetic, and molecular perspectives of plant-insect interactions with added emphasis on bioinformatic, genomic, and transcriptome analysis are comprehensively treated in this book. It presents the agro-ecological and evolutionary aspects of plant-insect interactions with an exclusive focus on the climate change effect on the resetting of plant-insect interactions. A valuable resource for biotechnologists, entomologists, agricultural scientists, and policymakers, the book includes theoretical aspects as a base toward real-world applications of holistic integrated pest management in agro-ecosystems.

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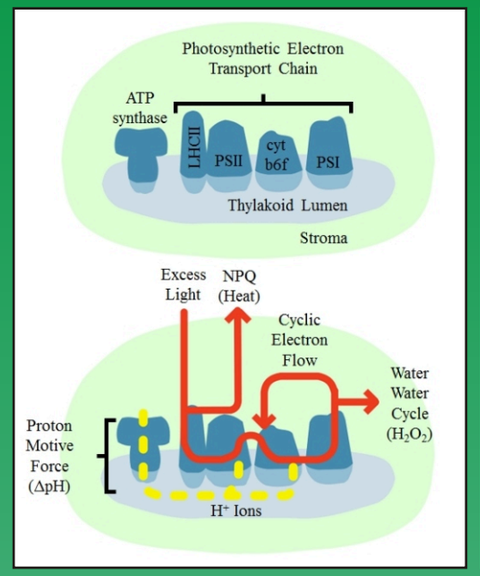
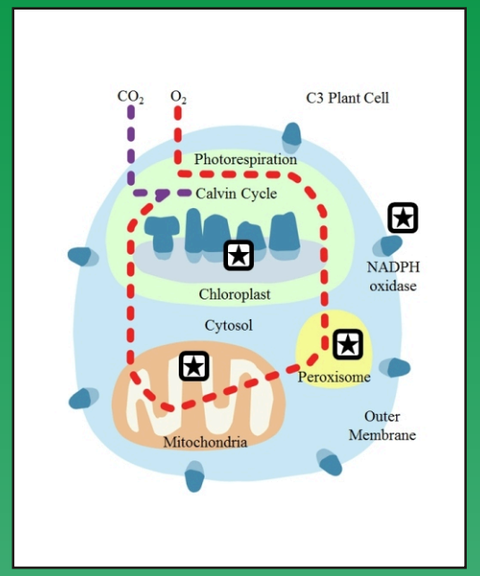
A Compendium for the Plant Biotechnologist
Editor Chandrakanth Emani





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Editor

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Preface

Insect is derived from Latin "insectum" means a "notched or divided body" and more literally is translated as "cut into". Though the term "cut into" refers more to the notched or cut up body segments of the insect, the word can be more apt referring to the herbivory that affects plants and crop systems in the ecological realm. Since the dawn of humanity, the over million insect species that have been described have been the most significant pests that affected crops, forests and fields, in some cases affecting mass human migrations. Some of the worst documented human catastrophes since the dawn of civilization were of insect pest attacks as in the grasshopper plagues of ancient Egypt, the devastating aphid attacks that wiped out French vineyards, and the Colorado beetle attack that caused the Irish potato famine.

The oldest fossils of insects as Tuft University entomologists recovered were 300 million year old carboniferous flying insect fossil including the definitive 396 million year old "Rhynie Chert" fossil that had an insect fossil related to the modern day silverfish insect speaks of their ancient evolution that paralleled those of plants. The diversity of the insect species in multiple ecological niches of the biosphere that followed showcases the innate ability to adjust in any kind of environment. This is the reason why almost half the biosphere's organisms are insects living in environments as varied as blazing deserts to tropical forests to the polar ice caps. It is no exaggeration to state that insects have literally gobbled up more food than humans themselves consumed since agricultural production began 10,000 years ago. This led to humans devising strategies to control the insect pests. What began with environmental friendly practices such as the flooding of rice fields by south India's farmers to drown insects, or the Chinese method of infesting lemon orchards with ants that ate the Vanessa butterflies led to the usage of vegetable derived nicotine or pyrethrum extracts followed by chemicals such as arsenic and copper sulfate opening the doors to insecticides and pesticides. The research that followed in the world of chemical agriculture reached its pinnacle with the Nobel Prize winning discovery of an effective insecticide, namely, the dichloro-diphenyl-trichloro ethane (DDT) by the Swiss chemist Herman Mueller.

The usage of DDT during second world war against the malaria mosquitoes extended to agricultural fields as it was seen as a holistic extermination tool against a vast array of plant insect pests. The initial euphoria in the form of increased agricultural yields was replaced by the nightmarish environmental contamination effects as DDT was found to be harmful to mammals, fishes, birds and humans. Entomologists since that time have turned their attention to research on the actual plant-insect interactions to design more holistic strategies that ultimately led to integrated pest management. The modern era of agriculture that saw the revolutions started by plant breeders later leading to the plant genetic engineering approaches primed the entomological researchers to explore the facets of plant-insect interactions in completely newer paradigms that spanned biochemistry, genetics and molecular biology.

The present volume seeks to review the biology of plant-insect interactions as a compendium to aid the plant biotechnologist bringing together the latest advances in the field in a comprehensive fashion covering the biochemical, genetic, molecular aspects with specific case studies in model crops. The book also touches on the more recent approach of exploring the plant-insect interactions in the climate change paradigm that offers a fresh approach to the time-tested strategy of integrated pest management.

It is hoped that the book will serve the needs of not only the plant biotechnologist, but could also serve as a ready reference to plant physiologists, biochemists, entomologists in both teaching and research endeavors.

Dr. Chandrakanth Emani

Contents

CHAPTER 1

Plant Protease Inhibitors and their Interactions with Insect Gut Proteinases

S.MD. Akbar,[1,2] *Jagdish Jaba*,[1] *Visweshwar Regode*,[1] *G. Siva Kumar*[1] and *H.C. Sharma*[1,3,*]

INTRODUCTION

Enzymes hydrolysing peptide bonds have some overlapping terms, these include, proteases, proteinases and peptidases (Barrett et al. 1998). The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB 1992) recommended peptidase as the general term for enzymes hydrolyzing peptide bonds, which is further divided into exopeptidases, which catalyse the cleavage of one or a few amino acids from N-/C-terminus, and endopeptidases, which cleave the internal peptide bonds of polypeptides. The term "protease" includes both exopeptidases and endopeptidases while "proteinase" designates only endopeptidases (Barrett et al. 1998). Proteolytic enzymes are extensively found in plants, animals and in microorganisms (Kenny 1999) with a major role involved in every aspect of their physiology and development. Proteases are highly specific to their substrate, and the specificity depends on the localization of the substrate and the proteolytic enzyme, and structural and chemical properties at the active site of the enzyme.

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Their mode of action varies among all families and groups of proteases. Some of them work individually, some work in cascades in cooperation with other proteases and some form complexes constituting an active proteolytic machine. In plants, various roles of proteolytic enzymes involves: removal of misfolded, modified, and/or mistargeted proteins; supply of amino acids during translation; maturation of zymogens and peptide hormones by partial cleavages; control of metabolism and homeostasis by altering the levels of key enzymes and regulatory proteins; and the cleavage of targeted signals from proteins prior to their final integration into organelles (Vierstra 1996). In insects, proteolysis allows digestion of wide range of food diet mediated by concerted action of several proteases and several of them such as trypsin, chymotrypsin, aminopeptidase, etc., have been characterized from a vast variety of insect pests till now (Anwar and Saleemuddin 2002; Sanatan et al. 2013; Akbar et al. 2017). The insect attack on plants triggers the production of a series of secondary metabolites; definsins, thionines, lectins, and protease inhibitors which altogether constitute the defensive armoury of plants (Buchmanan et al. 2002). Plant protease inhibitors are proteinacious in nature and inhibit insect gut proteases by binding tightly to the active site, forming an essentially irreversible complex. The inability to utilize ingested protein and to recycle digestive enzymes results in critical amino acid deficiency, which affects the growth, development and survival of the herbivore (Chougule et al. 2008). In this chapter, we aim to summarize the interactions between insect midgut proteases and the plant protease inhibitors induced as a result of insect attack.

Plant Protease Inhibitors

Plant proteinase inhibitors (PIs) play major role as potent defensive proteins against insect pests and pathogens. Diverse endogenous functions for these proteins has been proposed ranging from regulators of endogenous proteinases to storage proteins, but evidences for many of these roles is partial, or confined to limited examples. Plant PIs are small proteins generally present in high concentration in storage tissues, contributing up to 10% of the total protein content, they are also detectable in leaves in response to the attack of insects and pathogenic microorganisms (Ryan 1990). Many PIs act as defensive compounds against major insect pests either by direct assay or by expression in transgenic crop plants. The genes responsible for the production of PIs have been deployed in plants for the building of transgenic crop plants as a part of implications in integrated pest management programmes. Plant PIs are one of the

important candidates with highly proven inhibitory activity against insect pests and also known to improve the nutritional quality of food (Sharma et al. 2015). The role of PIs for plant protection was investigated as early as 1947, when Mickel and Standish (1947) ascertained that the larvae of few insects were unable to complete its biology normally on soybean products. Subsequently, the trypsin inhibitors present in soybean were observed to be toxic to the larvae of flour beetle *Tribolium confusum* (Lipker et al. 1954). The Protease inhibitor is the largest class of proteins that have undergone extensive investigations and consequently, their structure, properties, function and metabolism have been well documented. *In vitro* feeding trials using artificial diets containing the inhibitors have confirmed the protective role for protease inhibitors against several crop pests.

The detrimental effects of plant PIs to insect pests are accomplished by blocking insect midgut proteinases resulting in impaired protein digestion, which inhibits or at least delays (in the case of weak inhibitors) the release of peptides and amino acids from dietary protein. This impaired protein digestion therefore affects the insects' survival leading to lower growth rate and extended developmental period. The presence of inhibitor avoids the availability of nutrients in insects particularly sulphur containing amino acids, and thereby resulting in weak and stunted growth and in some cases it ultimately results in death (Gatehouse et al. 1992). The majority of proteinase inhibitors studied in plant kingdom originates from three main families namely Leguminosae, Solanaceae and Gramineae (Richardson 1991). These protease inhibitor genes have practical advantages over genes encoding for complex pathways, i.e., by transferring a single defensive gene from one plant species to another and expressing them from their own inducible or constitutive promoters thereby imparting resistance against insect pests (Boulter 1993). This was first demonstrated by Hilder et al. (1987) by transferring trypsin inhibitor gene from *Vigna unguiculata* to tobacco, which conferred resistance to wide range of insect pests including lepidopterans, such as *Heliothis* and *Spodoptera*, coleopterans such as *Diabrotica*, *Anthonomnous* and orthoptera such as Locusts.

Classification of Protease Inhibitors

PIs have been sorted into families, subfamilies and class based on sequence and reactive active site of the inhibitory domains. Based on sequence homologies of inhibitor domains, PIs have been classified into 48 families (Rawlings et al. 2004b). These inhibitor families have been found specific for each of the classes of proteolytic enzymes.

Serpin Family (Serine Protease Inhibitors)

The serpin family is the largest and the most widespread super family of PIs. Serpin-like genes have been identified in nearly all types of organisms, including viruses, bacteria, plants and animals (Law et al. 2006; Gettins 2002). Prokaryotes generally have a single serpin gene (Irving et al. 2002a). Inhibitors of serine proteinases have been described in many plant species, and are universal throughout the plant kingdom, with trypsin inhibitors being the most common type. Plants serpins are irreversible 'suicide' inhibitors and possess the molecular mass in the range of 39–43 kDa. The cleavage of an appropriate peptide bond in the reactive center loop of the inhibitor triggers a rapid conformational change so that catalysis does not proceed beyond the formation of an acyl-enzyme complex (Huntington et al. 2000). The majority of serpins inhibit serine proteases, but serpins that inhibit caspases (Ray et al. 1992) and papain-like cysteine proteases (Irving et al. 2002b) have also been reported. Plant serpins exhibit differing and mixed specificities towards proteases (Al-Khunaizi et al. 2002). Barley (*Hordeum vulgare*) serpin is a potent inhibitor of trypsin and chymotrypsin at overlapping reactive sites (Dahl et al. 1996a). Wheat (*Triticum aestivum*) serpins inhibit chymotrypsin and cathepsin G and have glutamic acid, lysine or arginine at P1 site (Roberts et al. 2003). Two oats (*Avena sativa*) serpins show specificity for chymotrypsin and/or elastase, and another one has specificity for trypsin and chymotrypsin at overlapping loop sites (Irving et al. 2002b,c). Squash serpin Cmps-1 also inhibits elastase at two overlapping sites (Ligoxygakis et al. 2003).

Plants do not use serine proteinases in processes involving large-scale protein digestion, and hence the presence of significant quantities of serpins with specificity towards these enzymes in plants cannot be used for the purpose of regulating endogenous proteinase activity (Reeck et al. 1997). Part of this bias can be accounted for by the fact that, mammalian trypsin is readily available and is the easiest of all the proteinases to assay using synthetic substrates, and hence is used in screening procedures. Because of these reasons the members of the serine class of proteinases have been the subject of intense research than any other class of PIs. All serine inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with a similar standard mechanism (Laskowski et al. 1980).

Serine proteinase inhibitors have anti-nutritional effect against several lepidopteran insect species (Applebaum 1985). Serine proteinases present in the midgut of insects, particularly those of Lepidoptera, were found to be inhibited by these serpins (Houseman et al. 1989). Yoo et al. (2000) have reported that feeding of purified serpin to aphids had no impact

on insect survival. These data suggest a more complex role for plant serpins other than insect pests defense mechanisms (Wieczorek et al. 1985; Yoo et al. 2000). Purified Bowman-Birk trypsin inhibitor (Brovosky 1986) at 5% of the diet inhibited growth of these larvae but SBTI (Kuntiz 1945), another inhibitor of bovine trypsin, was less effective when fed at the same levels. Broadway and Duffey (1986a) compared the effects of purified SBTI and potato inhibitor II (an inhibitor of both trypsin and chymotrypsin) on the growth and digestive physiology of larvae of *Heliothis zea* and *Spodoptera exigua* and demonstrated that growth of larvae was inhibited at 10% of the proteins in their diet. Trypsin inhibitors at 10% of the diet were toxic to larvae of the *Callosobruchus maculatus* (Gatehouse and Boutler 1983) and *Manduca sexta* (Shulke and Murdock 1983). Apart from their role in defense response to insect attack, serine protease inhibitors have also shown specificity to serine proteinases of broad range of organisms. Three pure trypsin inhibitors, SBTI, LBI and an egg white inhibitor (EWI) inhibited trypsins and chymotrypsins from 12 animal species with the wide range of variability (Sharma 2015). The buckwheat (*Fagopyrum sculentum*) trypsin/chymotrypsin inhibitor interferes with spore germination and mycelium growth of the tobacco brown-spot fungus *Alternaria alternata* (Dunaevskii et al. 1997). PIs from pearl millet inhibit growth of many pathogenic fungi including *Trichoderma reesei* (Joshi et al. 1998). Inhibitors that specifically inhibit proteolytic enzymes from microorganisms and not digestive proteases of animals are common in the plant, especially legume seeds. The inhibitors of the serine class of the enzymes secreted by *Bacillus subtilis* (subtilisins, or SIs) are found in seeds or vegetative tissues of many legume, cereal, and tuberous crops.

Structural analysis of serine protease inhibitors would greatly help in enzyme engineering of the native PIs to a potent form against the target pest species than the native PIs. X-ray crystallography structure proposed for the winged bean, *Psophocarpus tetragonolobus* Kunitz-type double headed alpha-chymotrypsin inhibitor showed 12 anti-parallel beta strands joined in a form of beta-trefoil with two reactive site regions (Asn 38-Leu 43 and Gln 63-Phe 68) at the external loops (Mukhopadhyay 2000). Indian finger millet (*Eleusine coracana*) bifunctional inhibitor of alpha-amylase/trypsin with 122 amino acids has shown five disulfide bridges and a trypsin binding loop (Gourinath et al. 2000).

Bowman-Birk Inhibitors (BBIs) Family

On the basis of sequence homology, this forms another family of serine PIs. The family is named after D.E. Bowman and Y. Birk, who were the first to identify and characterize a member of this family from soybean

(*Glycine max*) (Bowman 1946; Birk et al. 1963). The soybean inhibitor is now the most-well-studied member of this family and is often referred as the classic BBIs. The inhibitors have been found in legumes (Laing and McManus 2002; Tanaka et al. 1997) and in the grass family Poaceae (Odani et al. 1986). The inhibitors of this family are generally found in seeds, but are also wound-inducible in leaves (Eckelkamp 1993). They have been classified on the basis of their structural features and inhibitor characteristics. The BBIs from dicotyledonous plants consist of a single polypeptide chain with the molecular mass of 8 kDa. These are double-headed with two homologous domains each bearing a separate reactive site for the cognate proteases. These inhibitors interact independently, but simultaneously with two proteases which may be same or different (Raj et al. 2002; Birk 1985). The first reactive site in these inhibitors is usually specific for trypsin, chymotrypsin and elastase (Qi et al. 2005). The active site configuration in these inhibitors is stabilized by the presence of seven conserved disulfide bonds (Chen et al. 1992; Lin et al. 1993).

The BBIs are cysteine-rich proteins with inhibitory activity against proteases and are widely distributed in monocot and dicot species (Lin et al. 2006). BBIs from monocotyledonous plants are of two types. One group consists of a single polypeptide chain with a molecular mass of about 8 kDa having a single reactive site. Another group has a molecular mass of 16 kDa with two reactive sites (Tashiro et al. 1987, 1990; Prakash et al. 1996). It has been suggested that larger inhibitors have arisen from smaller ones by gene duplication (Odani et al. 1986). In the case of double-headed BBIs, it has been found that the relative affinity of binding of proteases is altered when one site is already occupied. Peanut (*Arachis hypogoea*) inhibitor has been found to exhibit no activity against chymotrypsin when preoccupied with trypsin and vice versa (Tur et al. 1972). In the same way, the activity of soybean BBIs decreases 100-fold when trypsin is bound at the other site (Gladysheva et al. 1999). The BBIs family of protease inhibitors contains a unique disulfide-linked nine-residue loop that adopts a characteristic canonical conformation (Bode and Hubr 1992). The loop is called protease-binding loop and binds the protease in a substrate-like manner (Lee and Lin 1995).

The trypsin subclass of serine protease inhibitors from legume seeds exhibit insecticidal effects against several crop pests belonging to the orders of Lepidoptera, Coleoptera and Orthoptera. Many of these inhibitors are products of multigene families with varying specificities towards different proteases. The cowpea trypsin inhibitor constitutes a larger gene family of four major iso-inhibitors. Three of the iso-inhibitors are specific for trypsin at each active site and the fourth is a trypsin-chymotrypsin bifunctional inhibitor (Sharma 2015).

Kunitz Family

The Kunitz inhibitors are the second major family of inhibitors which are widely distributed and often very abundant in seeds of leguminous plants and also occurs in other groups of plants including cereal seeds. On the basis of sequence homologies Kunitz-type inhibitors form a separate family. The members of this family are mostly active against serine proteases and may also inhibit other proteases (Laing and McManus 2002; Ritonja et al. 1990). The typical legume proteins are trypsin inhibitors of M_r about 21,000 Da with four cysteine residues that form two intra chain disulphide bonds and possess one reactive site. However, in the members of the legume sub family Mimosoideae, a proteolytic cleavage occurs between the third and fourth cysteine residues resulting in a heterodimeric protein comprising chains of M_r about 5,000 and 16,000 Da linked by a single disulphide bond. The inhibitors in this family are widespread in plants and have been described in legumes, cereals and in solanaceous species (Ishikawa et al. 1994; Laskowski and Kato 1980). A 20.5 kDa Kunitz-type trypsin inhibitor with antifungal activity has been reported from the roots of punce ginseng, *Pseudostellaria heterophylla* (Wang and Ng 2006). Kunitz-type PIs are also produced under stress, as has been found in potato tubers, *Solanum tuberosum* (Park et al. 2005; Ledoigt et al. 2006; Plunkett et al. 1982).

The Kunitz type trypsin inhibitor inhibits trypsin through interaction with a single site on the inhibitor and that is encoded by the KTi3 gene. Specificity of trypsin inhibitor is determined by the two amino acids residues, arginine and isoleucine, at the active site of the KTi protein; these amino acids are considered essential for inhibitor function, although arginine and serine are the active site residues in other inhibitors (Sharma 2015). However, not all the kunitz related proteins of legume seeds are proteinase inhibitors. The winged bean albumin-1, storage protein from *Psophocarpus tetragonolobus* L., accounts for about 15% of the total seed protein. It comprises 175 amino acid residues with a Mr of 19,333 and contains the single disulphide bond. It shows 38% and 28% sequence similarity with Kunitz inhibitors from soyabean and winged bean, respectively, but has no inhibitory activity (Kortt et al. 1989). The members of this family are mostly active against serine proteases and have been shown to inhibit trypsin, chymotrypsin and subtilisin (Laing and McManus 2002; Park et al. 2005), but they also inhibit other proteases including the aspartic protease cathepsin D and the cysteine proteinase papain. These inhibitors are canonical and form a tight complex with the target protease, which dissociates very slowly (Ritonja et al. 1990). It has been found that potato tubers treated with elicitors, jasmonic, salicylic

or arachidonic acids are able to excrete potatin and three chymotrypsin inhibitors (Ledoigt et al. 2006). Wounding and water stress promotes the secretion of two kinds of Kunitz-type PIs by potato tubers. These inhibitors are closely associated with other secreted polypeptides and would protect them against degradation by extracellular chymotrypsin-like protease. The secreted inhibitors could therefore interact with plant defense system (Valueva et al. 2001; Ledoigt et al. 2006).

Squash Inhibitors

Squash-family inhibitors have been described only in plants from many cucurbit families and form yet another family active against serine proteases (Lee and Lin 1995; Felizmenio et al. 2001). Seven serine PIs belonging to this family have been isolated and characterized from the seeds of wild cucumber, *Cyclanthera pedata* (Kuroda et al. 2001). Recently two different but inter-convertible (cis-trans isomers) inhibitors have been isolated and characterized from seeds of wax gourd, *Benincasa hispida* (Thumb) cogn. (Atiwetin et al. 2006). The members of this family consist of a small single peptide chain containing 28 to 30 amino acids with molecular mass of 3.0–3.5 kDa (Heitz et al. 2001). These inhibitors have three disulfide bridges and fold in a novel knottin structure (Hara et al. 1989). The small size of these inhibitors combined with potential activity against important biological molecules such as Hageman factor, human leucocyte elastase and cathepsin G (McWherter et al. 1989) has made them particularly attractive for studying proteinase and inhibitor interactions. Chemical synthesis of these inhibitors has created powerful tools for investigating their structural and functional relationship (Rolka et al. 1992). The structures of squash inhibitors and inhibitor-proteinase complexes have been determined by X-ray crystallography and NMR spectroscopy (Thaimattam et al. 2002).

Cereal Trypsin/Amylase Inhibitors

The members of this family have serine proteinase inhibitory activity and/or amylase inhibitory activity (Gourinath et al. 2000). A large number of inhibitors in this family have only amylase inhibitory activity; however, inhibitors from barley (*H. vulgare*), rye (*Secale cereale*) and tall fescue (*Festuca arundinacea*) are active against trypsin (Odani et al. 1983). Maize (*Zea mays*) and ragi (*Elusine coracana*) inhibitors show dual activities and can inhibit serine proteinases as well as amylase (Mahoney et al. 1984; Shivraj and Pattabiraman 1981). The cereal trypsin/amylase inhibitors consist of a single polypeptide chain containing five disulfide bonds with a molecular mass of about 13 kDa (Christeller and Liang 2005). The structure of the

ragi inhibitor solved by NMR spectroscopy and that of its complex with yellow mealworm, *Tenebrio molitor* amylase by x-ray crystallography has shown that the proteinase-binding loop adopts a canonical conformation (Strobl et al. 1998).

Mustard (Sinapis) Trypsin Inhibitor (MSI)

These are small single polypeptide chain inhibitors with the molecular mass of about 7 kDa, found in the family Cruciferae and form yet another family of serine PIs (Laing and McManus 2002; Menengatti et al. 1992). These inhibitors have been isolated and characterized from a number of species including white mustard, *Sinapis alba,* and tape, *Brassica napus* (Ascenzi et al. 1999; Volpicella et al. 2000). These inhibitors are expressed in seeds during their development and are also wound-inducible (Ceci et al. 1995; De Leo et al. 2001). The inhibitors form a tight binding complex with trypsin and apparently follow the standard mechanism of inhibition as that of serpins (Ceciliani et al. 1994).

Potato Type I Protease Inhibitors (PI 1)

The inhibitors of this family are widespread in plants and have been described in many species including potato tubers (Ryan and Balls 1962), tomato fruit (Margossian et al. 1988; Wingate et al. 1989), squash phloem exudates (Murray and Christeller 1995) and in tomato leaves in response to wounding (Lee et al. 1986). These inhibitors have the molecular mass of 8 kDa and are generally monomeric. While the inhibitors from cucurbit and potato tubers contain a single disulphide bond, the inhibitors in this family lack any disulphide bridges (Cai et al. 1995). The inhibitory mechanism in this family is considered to fit the standard model.

Potato Type II Protease Inhibitors (PI 2)

The members of this group have been reported only from the members of Solanaceae family. Initially characterized from potato tubers (Christeller and Liang 2005) and these inhibitors have been found in leaves, flowers, fruit and phloem of other solanaceaous species (Iwasaki et al. 1971; Pearce et al. 1993). Among the PIs, the wound-inducible inhibitors from potato and tomato represent a unique group with insecticidal properties due to several interesting features of these proteins and their encoding genes. An analysis of these inhibitors and genes has shown that they are composed of multiple repeat units varying between one and eight (Antcheva et al. 2001; Miller et al. 2000; Choi et al. 2000). They comprise a non-homologous gene family in which members have been identified

mainly from the solanaceous plants. Among them, potato inhibitor I and II, tomato protease inhibitor I and II have been well characterized. The unique and most striking feature of their encoding genes are the presence of introns, two each in inhibitor I genes and one in the gene encoding potato inhibitor II. In fact, they are the only protease inhibitor genes reported so far to contain introns. In potato alone, a mixture of one or more inhibitors of protease inhibitor I and at least three forms of inhibitor II have identified. In addition, homologs of the inhibitor have been found in some non-solanaceous plants like alfalfa, broad bean, clover, cowpea, cucumber, french bean, grape, squash, strawberry, barley and buckwheat.

In leaves of tomato and potato, they are expressed constitutively at low levels during plant growth and development. In response to wounding by insects or other mechanical damage, their concentration increases dramatically even in the unwounded leaves of the same plant and within a few hours of injury, their levels often exceed 10% of total soluble proteins. In potato tubers, they accumulate throughout the course of tuber development and represent a substantial fraction of the soluble protein. Thus, unlike other plant protease inhibitor gene, these genes are regulated environmentally as well as developmentally and their expression is believed to be under a complex control involving several *cis* and *trans*-acting factors making them excellent models for study of plant gene regulation. A low molecular-mass inhibitor of this family has been found to be constitutively present in Jasmme tobacco (*Nicotiana alata*) flowers (Atkinson et al. 1993). Six small wound-inducible proteinase inhibitors of this family have been reported from tobacco leaves (Pearce et al. 1993). Inhibitors of this family have been reported to inhibit chymotrypsin, trypsin, elastase, oryzin, Pronase E and subtilisin (Antcheva et al. 2001).

Cysteine Protease Inhibitors (The Cystatin Superfamily)

The members of these families inhibit the activity of cysteine proteases and are called cysteine PIs or cystatins. The cystatin super family (CYS) is composed of several families and includes proteins that are related in structure and function to cysteine proteinase inhibitors. They were first described in egg white and referred to as 'chicken eggwhite cystatin' (Colella et al. 1989). They are widely distributed in plants, animals and microorganisms (Oliveira et al. 2003). Most cysteine proteinase inhibitors have been found in animals, but several have been isolated from plant species as well including pineapple, potato, corn, rice, cowpea, mungbean, tomato, wheat, barley, rye and millet. Cysteine proteinases are not secreted as intestinal digestive enzymes in higher animals, but are found in the midguts of several families of Hemiptera and Coleoptera where they appear to play important roles in the digestion of food proteins. In a

study of the proteinases from the midguts of several members of the order coleoptera, 10 of 11 beetle species representing 11 different families had gut proteinases that were inhibited by p-chloromercuribenzene sulphonic acid (PCMBS), a potent sulphydryl reagent, indicates that the proteinases are in the pH range of the insect gut that usually possess cysteine proteinases. Expression of the PI genes of these inhibitors are usually limited to specific organs or to particular phases during plant growth, such as germination (Botella et al. 1996), early leaf senescence (Huang et al. 2001) and drought (Van der-Vyver et al. 2003; Pernas et al. 2000). Wounding or treatment with methyl jasmonate evokes a similar pattern of gene expression. Further, the cytosolic localization of these inhibitors also suggests that they are involved in plant defense against insects (Zhao et al. 1996).

The rice cysteine proteinase inhibitors are the most studied of all the cysteine PIs which is proteinaceous in nature and highly heat stable (Abe et al. 1987). Phytostatins from various plants inhibit the activity of gut cysteine proteinases involved in protein digestion in the gut of various members of the Coleoptera (beetles) attacking these plants, and thus play a role in the exogenous defense system of these plants (Oliveira et al. 2003). *Oryza cystatin* is found to prevent the growth of rice weevil, *Sitophilus oryzae*, by inhibiting the cysteine proteases in the gut of this organism (Hosoyama et al. 1994). The rice cystatins have been reported to confer resistance against potyviruses in transgenic tobacco and sweet-potato plants (Campos et al. 1999). Two extracellular cysteine protease inhibitors (ECIP-1 and ECIP-2) isolated from species of the unicellular green alga *Chlorella* seem to have a role in protecting the cells from attacks by viruses and insects (Ishihara et al. 1999, 2000). Cystatins have also been characterized from potato, ragweed, cowpea, papaya and avocado. Cysteine PIs from pearl millet (*Pennisetum glaucum*) inhibits the growth of many pathogenic fungi, including *Trichoderma reesei* (Joshi et al. 1998). These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests and pathogen. Transgenic rice expressing maize cystatin has been shown to exhibit enhanced resistance against insect predation (Irie 1996).

Zeins and maize proteinases are inhibited by maize cystatins, suggesting a role for these inhibitors in the endogenous defense mechanism (Steller 1995; Hoorn and Jones 2004). Phytostatins are involved in the control of endogenous cysteine proteinases during maturation and germination of seeds (Abe and Arai 1991) and play a role in the apoptosis required in plant development and senescence (Solomon et al. 1999). Oryzacystatins have been shown to inhibit the cysteine proteinases that are produced during seed germination (Watanabe et al. 1991).

The over-expression of cystatin in soybean cell suspensions blocked programmed cell death (PCD) (Solomon et al. 1999). The over-expression

of a cystatin that inhibits papain activity in *Arabidopsis* cell cultures blocked cell death in response to avirulent bacteria and nitric oxide (Belenghi et al. 2003). The over-expression of this inhibitor in tobacco plants blocked the hypersensitive response induced by avirulent bacteria (Hoorn and Jones 2004; Belenghi et al. 2003). These cysteine protease inhibitors are grouped into four families based on sequence relationships, molecular mass and disulfide-bond numbers and arrangements (Barrett 1987).

Family-1 Cystatins (Stefin Family)

The members of this group have a molecular mass of about 11 kDa. They are generally present in the cytosol and are devoid of any carbohydrate groups and disulfide bonds (Stato et al. 1990; Machleidt et al. 1983).

Family-2 Cystatins (Cystatin Family)

These inhibitors consist of proteins having 120–126 amino acids with molecular mass of 13.4–14.4 kDa. These inhibitors contain two disulphide bonds but are devoid of any carbohydrate groups (Grzonka et al. 2001). They also contain a signal sequence and are known to be secreted (Abrahanson et al. 1987). All the family-2 cystatin inhibitors contain a conserved tripeptide sequence, Phe-Ala-Val near the C-terminus, and a conserved dipeptide, Phe-Tyr, near the N-terminus. These conserved sequences are important in binding to the target proteases (Machleidt et al. 1983; Turk et al. 1997).

Family-3 Cystatins (Kininogen Family)

These inhibitors are glycoproteins and are of three different types, High Molecular Weight kininogens (HMW) with a molecular mass of 120 kDa, Low Molecular Weight kininogens (LMW) with molecular mass ranging between 60 and 80 kDa, and a third type, T kininogens with a molecular mass of 68 kDa. These proteins contain tandem domains that result from gene duplication of the family-2 cystatins. These proteins are also secreted and play key roles in blood coagulation (Otto and Schirmeister 1997; Salvesen et al. 1986). Family 1 and 3 cystatins contain a conserved pentapeptide sequence, Gln-Val-Val-Ala-Gly, and the family-2 members have the homologous peptide, Gln-X-Val-Y-Gly, in which X and Y represent any amino acid (Habib and Fazilil 2007).

Family-4 Cystatins (Phytocystatins)

This family includes nearly all the cysteine PIs described in plants. They have been identified in rice (Abe et al. 1987a,b), maize (Abe et al. 1992),

soybean (Hines et al. 1991; Botella et al. 1996), apple (*Malus*) fruit (Ryan et al. 1998), carnation (*Dianthus caryophyllus*) leaves (Kim et al. 1999) and several other monocotyledonous and dicotyledonous plants (Brown and Dziegielewska 1997; Pernas et al. 1998; Sakuta et al. 2001). Celostatin, a cysteine PI from crested cock's comb (*Celosia cristata*) has recently been cloned and characterized (Gholizadeh et al. 2005). Phytocystatins have sequence similarity to stefins and cystatins, but do not contain free cysteine residues (Fernandes et al. 1993; Zhao et al. 1996). However, the unique feature of this superfamily is a highly conserved region of the G58 residue, the glu-val-val-ala-gly (QVVAG) motif and a pro-trp (PW) motif. The studies on the papain inhibitory activity of Oryzacystatin and its various truncated forms have identified the conserved QVVAG motif as a primary region of interaction between the inhibitor and its cognate enzyme. The PW motif is believed to act as a cofactor (Arai et al. 1991; Abe et al. 1988). Phytocystatins, based on protein structure, have been divided into two groups, one group consists of single-domain proteins and includes most these inhibitors (Abe et al. 1987a,b; Pernas et al. 1998), another group contains multiple-domains and includes the cysteine PIs isolated from potato tubers and tomato leaves (Walsh and Strictland 1993; Bolter 1993). Plant cysteine PIs are encoded by gene families (Fernandes et al. 1993) and show different expression patterns during development and defense response to biotic and abiotic environmental stress (Felton and Korth 2000). The expression is usually limited to specific organs or to specific phases during development, such as germination (Botella et al. 1996), early leaf senescence (Huang et al. 2001) cold and salt stress (Van der-Vyver et al. 2003; Pernas et al. 2000).

Aspartyl and Metallocarboxypeptidase Inhibitors

Aspartyl PIs have been isolated from sunflower, barley and cardoon (*Cynara cardunculus*) flowers are named as cardosin A (Park et al. 2000; Kervinen et al. 1999; Lawrence and Koundal 2002; Mares et al. 1989; Wolfson and Murdock 1987). In species of six families of the order Hemiptera, aspartic proteinases (cathepsin D-like) were found along with cysteine proteinases (Houseman 1983). The cathepsin D inhibitor, an aspartyl PI described in potato tubers shares considerable amino acid sequence homology with soybean trypsin inhibitor. It is a 27 kDa protein and inhibits serine proteases trypsin and chymotrypsin in addition to the aspartyl protease cathepsin D, but does not inhibit pepsin, cathepsin E and rennin, which are all aspartyl proteases (Lawrence and Koundal 2002). The inhibitor also accumulates in potato leaf tissues along with serine proteinase inhibitor I and II proteins in response to wounding. Thus, the inhibitors accumulated in the wounded leaf tissues of potato have the capacity to inhibit all

the five major digestive enzymes, i.e., trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B of higher animals and many insects (Hollander 1985). Pepstatin, a powerful and strong inhibitor of aspartyl proteases has been shown to inhibit proteolysis of the midgut enzymes of Colorado potato beetle (*Leptinotarsa decemlineata*) (Wolfson and Murdock 1987).

Plants contain two families of metalloproteinase inhibitors, the metallocarboxypeptidase inhibitor family in potato and tomato plants (Graham and Ryan 1997; Rancour and Ryan 1968) and a cathepsin D inhibitor family in potatoes (Keilova and Tomasek 1976). The inhibitors that bind to metallocarboxypeptidases have been identified in solanaceous plants, in the medicinal leech (*Hirudo medicinalis*), in the intestinal parasite roundworm *Ascaris sum*, in the blood tick *Rhipicephalus bursa* and in rat and human tissues (Arolas et al. 2005; Homandberg et al. 1989; Reverter et al. 1998; Normant et al. 1995; Liu et al. 2000). These inhibitors are small peptide inhibitors consisting of 38–39 amino acid residues and have the molecular mass of about 4.2 kDa (Hass et al. 1975; Hass and Hermodson 1981). These inhibitors are polypeptides (4 kDa) inhibit strongly but competitively to a broad spectrum of carboxypeptidases from both animals and microorganisms, but do not inhibit serine carboxypeptidases from yeast and plants (Havkioja and Neuvonen 1985). A metallocarboxypeptidase inhibitor is found to accumulate in potato tuber tissues during development, along with the potato inhibitor I and II families of serine PIs. The inhibitor also accumulates in potato leaf tissues, along with the inhibitors of other families, as a response to wounding (Ryan 1990).

Insect Resistant Transgenic Plants Expressing PIs

Since the economically important orders of insect pests namely Lepidoptera, Diptera and Coleoptera use serine and cysteine proteinases in their digestive system to degrade proteins in the ingested food and efforts have generally been directed at genes encoding PIs active against these mechanistic classes of proteases for developing transgenic plants. The PI genes have been particularly utilized in developing transgenic plants resistant to insect pests and/or pathogen by transferring a single defensive gene that can be expressed from the wound-inducible or constitutive promoters of the host (Boulter 1993). Several transgenic plants expressing PIs have been produced in the last 15 years and tested for enhanced defensive capabilities with particular efforts directed against insect pests (Valueva et al. 2001).

The PI gene coding for cowpea trypsin inhibitor (CpTi) was the first to be successfully transferred and produced transgenic tobacco with

significant resistance against tobacco hornworm, *Manduca sexta* (Hilder et al. 1987). The efficiency of transgenic tobacco plants expressing CpTi was also tested against armyworm, *Spodoptera litura,* in feeding trials under laboratory conditions. Reduction of 50% biomass was observed in the larvae fed on transgenic leaves expressing 3–5 µg of CpTi/g of fresh leaves (Sane et al. 1997). Potato PI-II gene from potato was introduced into several japonica rice varieties to produce transgenic rice plants shown to be insect resistant in greenhouse trials. Wound-inducible PI-II promoter with the first intron of rice actin I gene could give high-level expression of PI-II gene in transgenic rice plants were resistant to pink stem borer, *Sesamia inferens* (Duan et al. 1996).

Bean α-amylase inhibitor I in transgenic peas, *Pisum sativum*, provided complete protection from pea weevil, *Bruchus pisorum*, under field conditions (Roger et al. 2000). When both soybean BBI and Kunitz inhibitors were introduced and expressed in sugarcane, *Saccharum officinarum*, the growth of neonate larvae of sugarcane borer, *Diatraea saccharalis* feeding the leaf tissues was significantly retarded as compared to larvae feeding on leaf tissues from untransformed plants (Falco and Silva 2003). The transgenic wheat, *T. aestivum*, carrying barley trypsin inhibitor gene (BTI) showed a significant reduction of infestation with Angoumois grain moth, *Sitotroga cerealella*. However, only early-instar larvae were inhibited in transgenic seeds and expression of BTI protein in transgenic leaves did not have a significant protective effect against leaf-feeding insects (Altpeter et al. 1999).

The PIs also exhibited a very broad spectrum of activity against pathogenic nematodes. CpTi inhibited the growth of nematodes, *Globodera tabacum*, *G. pallida* and *Meloidogyne incognita* (Williamson and Hussey 1996). Transgenic potato expressing two cystatin genes developed resistance to a nematode, coleopteran insects (Cowgill et al. 2002) and transgenic rape plants expressing rice cystatin 1 were resistant to aphid (Rahbe et al. 2003). Recently, protease inhibitors have also been used to engineer resistance against viruses in transgenic plants (Ussuf et al. 2001). The presence of anti-fungal and anti-feeding activity on a single protein explored a new possibility of raising a transgenic plant resistant to pathogens, as well as pests by transfer of a single CPI gene. Pearl millet cysteine protease inhibitor (CPI) has been found to possess anti-fungal activity in addition to its antifeedant activity against insects (Joshi et al. 1998). Expression of Oryzacystatin, the rice cysteine proteinase inhibitor, into the tobacco plant induced significant resistance against two important potyviruses, tobacco etch virus (TEV) and potato virus Y (PVY). These results suggest that plant cystatins can be used against different potyviruses and potentially also against other viruses whose replication involves cysteine proteinase activity (Campos et al. 1999).

These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests. Further, the transformation of plant genomes with PI-encoding cDNA clones appears attractive not only for the control of plant pests and pathogens, but also as a means to produce PIs, useful in alternative systems and the use of plants as factories for the production of heterologous proteins. A list of transgenics expressing plant PIs are included in Table 1.

Plant PIs in Defense and Limited Success as Insecticides

Plants PIs vary in protein primary sequence and tertiary structure to act with all mechanistic protease groups. A new PI categorization system has been showed with the increasing availability of sequence data and 3-D structural information, gradually replacing the previous categorization based on protease specificity (Rawlings et al. 2004; Jongsma and Beekwilder 2011). Although many PIs are minor proteins having a single inhibitory domain, it is not uncommon for PIs to contain two or more inhibitor units. Potato multi cystatin, for example, has eight tandem cystatin domains (Walsh and Strictland 1993).

The general mode of action of PI molecules includes inhibition of protein digestive enzymes in insect guts resulting in amino acid deficiencies which lead to delayed developmental growth, increased mortality, and/or reduced fecundity (Gatehouse 2011). The adverse effects of dietary PIs on insects may be more complex actions than a simple decrease in the proteolytic activity of the digestive enzymes complex. Feedback mechanisms in response to dietary PIs were suggested to lead to the hyper production of proteases to counterbalance for the loss of activity, causing the declining trends of essential amino acids. The imposed nutritional stress would later retard insect growth and development (Schechter and Berger 1967; Broadway and Duffey 1986). In addition to the direct inhibitory effect on proteolytic enzyme complexes, plant PIs may function in other processes. For instance, PIs inhibited normal development of cereal aphids, although a relatively higher concentration of free amino acids present in the sap it could not prevent the growth impairment caused by PIs, thus indicates the indirect effects on the aphid rather than inhibiting food protein digestion (Pyati et al. 2011).

Although plant PIs are an important component in insect pest management, but attempts to use single PIs in transgenic crops has very limited success. Since the initial effort in expressing a cowpea trypsin inhibitor in tobacco, *Nicotiana tabacum* (Hilder et al. 1987), transgenic plants holding foreign PI genes of various types have been developed in a range of plants, including wheat (*T. aestivum*), rice (*Oryza sativa*), cotton (*Gossypium hirsutum*) and *Arabidopsis thaliana* (Chapman 1988; Mosolov and

Table 1. List of few transgenic plants expressing plant PIs that have been developed and tested for their effectiveness on the growth and development of insect pests.

Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
Arabidopsis thaliana serpin 1 [AtSerpin1]	alpha-1-peptidase inhibitor	Chymotrypsin	*Arabidopsis*	*Spodoptera littoralis*	38% biomass reduction after feeding for 4 days (Alvarez-Alfageme et al. 2011)
Barley trypsin inhibitor [BTI]	Cereal Trypsin inhibitor	Trypsin	Tobacco	*Spodoptera exigua*	29% reduction in survival (Altpeter et al. 1999)
			Wheat	*Sitotroga cerealella*	No effect on growth or mortality (Lara et al. 2000)
Bovine pancreatic trypsin inhibitor [BPTI]	Kunitz (animal)	Trypsin, chymotrypsin, plasmin, kallikreins	Tobacco	*Spodoptera exigua*	Reduced trypsin activity; induced leucine, aminopeptidase and carboxypeptidase A activities; chymotrypsin, elastase, and carboxypeptidase B proteases not affected (Lara et al. 2000)
			Sugarcane	*Scirpophaga excerptalis*	Significant reduction in weight (Christy et al. 2009)
Bovine spleen trypsin inhibitor [SI]	Kunitz (animal)	Trypsin, chymotrypsin	Tobacco	*Helicoverpa armigera*	Reduced survival and growth (Christy et al. 2009)

Table 1 contd. ...

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Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
Cowpea trypsin inhibitor [CpTI]	Bowman-Birk	Trypsin	Tobacco	*Heliothis virescens*	Increased mortality (Hilder et al. 1987)
			Tobacco	*Helicoverpa zea*	Increased mortality (Hoffmann et al. 1992)
			Rice	*Chilo suppressalis-Sesamia inferens*	Growth not monitored (Xu et al. 1996)
			Potato	*Lacanobia oleracea*	45% biomass reduction (Gatehouse et al. 1997)
			Tobacco	*Spodoptera litura*	50% biomass reduction (Sane et al. 1997)
			Potato	*Lacanobia oleracea*	Decreased weight and delayed development (Bell et al. 2001)
Giant taro proteinase inhibitor [GTPI]	Kunitz (plant)	Trypsin, chymotrypsin	Tobacco	*Helicoverpa armigera*	Decreased growth, no increase in mortality (Wu et al. 1997)

Mustard trypsin inhibitor 2 [MTI-2]	Brassicaceae proteinase inhibitor	Trypsin, chymotrypsin	Tobacco, *Arabidopsis* and oilseed rape	*Spodoptera littoralis*	Increased mortality; surviving larvae up to 39% smaller after 10 days (De Leo et al. 1998)
				Mamestra brassicae, Plutella xylostella, Spodoptera littoralis	*P. xylostella:* 100% mortality on *Arabidopsis*; high mortality & delayed development on oilseed rape. *M. brassicae:* increased mortality & weight of survivers on *Arabidopsis* and tobacco, no effect on oilseed rape. *S. littoralis*: delay in development on oilseed rape (De Leo et al. 2001)
			Tobacco	*Spodoptera littoralis*	No effect on growth; reduction in fertility (De Leo and Gallerani 2002)
			Oilseed rape	*Plutella xylostella*	Reduction in survival and weight (Ferry et al. 2005)
Nicotiana alata protease inhibitor [NaPI]	Proteinase inhibitor II	Trypsin, chymotrypsin	Tobacco	*Helicoverpa punctigera*	Decreased weight; increased mortality (Heath et al. 1997)
			Tobacco and peas	*Helicoverpa armigera*	Increased mortality; delayed growth (Charity et al. 1999)
			Royal Gala' apple	*Epiphyas postvittana*	Larval and pupal weights reduced; developmental abnormalities (Maheswaran et al. 2007)
			Cotton	*Helicoverpa armigera*	A higher number of cotton bolls were recorded in plants expressing NaPI and a PotI inhibitor from potato, StPin1A (Dunse et al. 2010)

Table 1 contd. ...

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Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
Potato inhibitor II [Pin II, PPI-II, Pot II, PI-II]	Proteinase inhibitor II	Trypsin, chymotrypsin, oryzin, subtilisin, elastase	Tobacco	*Manduca sexta*	Growth retarded (Johnson et al. 1989)
			Tobacco	*Chrysodeixis eriosoma, Spodoptera litura, Thysanoplusia orichalcea*	*C. eriosoma* larvae grew slower; *S. litura and T. orichalcea* growth either unaffected or enhanced (McManus et al. 1994)
			Tobacco	*Spodoptera exigua*	Growth not affected (Jongsma et al. 1995)
			Rice	*Sesamia inferens*	Decreased weight (Duan et al. 1996)
			Brassica napus	*Plutella xylostella*	Lowered growth rates however more plant tissue consumed (Winterer and Bergelson 2001)
			Tomato	*Heliothis obsoleta*	Increased mortality and decreased weight on homozygous plants expressing PI-II and potato carboxypeptidase inhibitor (PCI), opposite effect on hemizygous plants (Abdeen et al. 2005)
Solanum americanum proteinase inhibitor [SaPIN2a]	Proteinase inhibitor II	Trypsin, chymotrypsin	Tobacco	*Helicoverpa armigera, Spodoptera litura*	Reduction in larval weight and pupation rate (Luo et al. 2009)

Soybean Kunitz trypsin inhibitor [SBTI, SKTI]	Kunitz (plant)	Trypsin, chymotrypsin, kallikrein, plasmin	Poplar	*Clostera anastomosis, Lymantria dispar*	Mortality and growth not Significantly affected (Confalonie et al. 1998)
			Potato	*Lacanobia oleracea*	Survival and growth decreased by 33% and 40% respectively after 21 days (Gatehouse et al. 1999)
			Tobacco	*Spodoptera litura*	Increased mortality and Delayed development (McManus et al. 1999)
			Tobacco	*Helicoverpa armigera*	Development unaffected (Nandi et al. 1999)
			Tobacco and potato	*Spodoptera littoralis*	High mortality on tobacco and up to 50% weight reduction on potato (Marchetti et al. 2000)
			Sugarcane	*Diatraea saccharalis*	Increased mortality; retarded growth (Falco et al. 2003)
Soybean Bowman-Birk trypsin inhibitor [SBBI]	Bowman-Birk	Trypsin, chymotrypsin	Sugarcane	*Diatraea saccharalis*	Growth severely retarded (Falco et al. 2003)
			Cauliflower	*Plutella xylostella, Spodoptera litura*	Increased mortality (Duan et al. 1996)
			Tobacco	*Spodoptera litura*	Growth and survival severely retarded (Yeh et al. 1997)

Table 1 contd. ...

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Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
Sweet potato trypsin inhibitor [SWTI, Sporamin]	Kunitz (plant)	Trypsin	Tobacco	*Helicoverpa armigera*	Increased mortality and delayed growth and development in larvae on plants expressing sporamin and a phytocystatin from taro, CeCPI (Senthilkumar et al. 2010)
			Brassica	*Plutella xylostella*	Survival rate and body mass was significantly lower in larvae fed plants. Expressing sporamin and chitinase (Liu et al. 2011)
Tomato inhibitor I [Tom1]	Proteinase inhibitor I	Chymotrypsin, subtilisin, trypsin	Tobacco	*Manduca sexta*	Little effect on growth (Johnson et al. 1989)
Tomato inhibitor II [TPI-II]	Proteinase inhibitor II	Chymotrypsin, trypsin, subtilisin	Tobacco	*Manduca sexta*	Growth retarded (Johnson et al. 1989)
Beta vulgaris serine proteinase inhibitor gene (BvSTI)			*Nicotiana benthamiana*	*Spodoptera frugiperda, Spodoptera exigua, Manduca sexta, Heliothis virescens* and *Agrotis ipsilon*	Reductions in larval weights in *S. frugiperda, S. exigua* and *M. sexta*. Developmental abnormalities of the pupae and emerging moths (Johnson et al. 1989)

Valueva 2008). However, no PI-transgenic plants have made commercially available, despite the detrimental effects of the PIs on insect pests. Under selection pressure, insects appear to develop resistance to many defense genes including those encoding PIs. Some even overcompensate for the nutritional hindrance by consuming more transgenic plant material and gaining more weight than with non-transgenics (De Leo et al. 1998).

The PIs undoubtedly form a significant component of a multi-mechanistic defense system used by plants. However, the remarkably flexible physiological responses displayed by insects facilitate their adaptation to the PI-based plant defense. Consequently, non-plant PIs were explored as a more effective measure to avoid the insufficiency of inhibition of plant PIs due to specific co-evolutionary interactions (Harsulkar et al. 1999). Spit et al. (2012) designed an inhibitor mixture based on the total midgut protease profile of the desert locust, *Schistocerca gregaria*. The mixture contains pacifastin-related inhibitors that are of non-plant origin with maximal *in vitro* inhibition of the trypsin- and chymotrypsin-like proteolytic activity of the *S. gregaria* midgut. Pacifastin family members have been found in all arthropods. Those that have been characterized are derived from precursor polypeptides consisting of an N-terminal signal sequence followed by a variable number of inhibitor domains (Breugelmans et al. 2009). The pacifastin-related inhibitor mixture resulted in greater suppression of insects compared with plant-derived inhibitors in feeding assays. However, the inhibitory effect gradually waned and insects recovered within a few days from the growth impediment.

A conjunct search for novel PIs apparently has resulted in little success. It has gradually become clear that the degree to which proteolytic digestion in an insect vulnerability is dependent not only on the PI's defensive mechanism but also on the insect's response. Fundamental knowledge of insect counter defense is a prerequisite for making use of PI and other anti-insect molecules for producing the next generation of insect-resistant transgenic plants.

Mechanism of PI Toxicity

The toxicity of the plant PIs depends upon the structural compatibility with the protease and the physiological conditions of gut of the target organism (e.g., pH) and also the quality and quantity of PI ingested (Broadway 1995). The mechanism of binding of the plant PIs to the insect proteases appears almost similar with all the classes of inhibitors (Laskowski et al. 1980). The inhibitor binds at the active site of the enzyme forming an enzyme-inhibitor complex with a very low dissociation constant (10^{7} to 10^{14} M at neutral pH values) thereby efficiently blocking the active site. The inhibitor therefore directly mimics a normal substrate of the enzyme

thereby blocking the usual enzyme mechanism of cleaving the peptide bond (Walker et al. 1998). Sometimes, a binding loop from the inhibitor projects from the surface of the molecule and contains a cleavable peptide bond for the enzyme, but the cleavage do not interfere the interaction between enzyme and the inhibitor (Terra et al. 1996; Walker et al. 1998).

Conclusion

Plant PIs are key players in the endogenous defense system as they help regulate and balance protease activities. These inhibitors are also important participants in the exogenous defense. The importance of PIs has been realized for some time now, and many transgenic plants over expressing different PIs have been produced with resistance against different insects and/or pathogenic organisms. This is, however, yet to be fully appreciated, and it can have important consequences beyond their recognized scope. These inhibitors can also interfere with the life cycle of many viruses and may help prevent many viral disorders. Although plant PIs have been isolated and characterized from many sources, and that the natural inhibitors have been made available through transgenic plants over expressing specific inhibitors with the potential for the natural inhibitors in agriculture is enormous, awaiting full-scale exploration to combat the insects' counter adaptations developed against them for sustainable crop production.

Insect Gut Proteinases

Insects generally use different types of digestive enzymes to digest a wide range of food diets, including polymeric molecules, which are secreted by midgut's epithelial cells (Terra and Ferreira 1994; Terra et al. 1996). Some carbohydrases and proteases can break down the carbohydrates and proteins into absorbable elements in midgut, respectively (Terra 1990). The proteinases are a noteworthy group of hydrolytic enzymes in insects and are included in digestive processes, proenzyme activation, freedom of physiologically dynamic peptides, supplement initiation, and aggravation forms among others (Neurath 1984). The proteinases are characterized by their mechanisms of catalysis: (1) serine proteinases; (2) cysteine proteinases; (3) aspartic proteinases; and (4) metalloproteinases (Bode and Huber 1992). For a proficient management of pest control through proteinase inhibitor transgenes, it is basic to know the sort of catalysts present in the gut of insects and pests. The two noteworthy proteinase classes in the digestive systems of phytophagous insects are the serine and cysteine proteinases (Haq et al. 2004). Murdock et al. (1987) completed a detailed investigation of the midgut proteins of different

pests having a place with Coleoptera, while Srinivasan et al. (2006) have covered the midgut enzymes of different pests having a place with Lepidoptera. Serine proteases are known to command the larval gut condition and add to around 95% of the aggregate stomach related action in Lepidoptera, though the Coleopteran species have a more extensive scope of predominant gut proteinases.

Serine Proteinases

Serine proteases (SPs) in the chymotrypsin (S1) family constitute one of the biggest gene groups of multifunctional enzymes that assume essential parts in different physiological procedures, including assimilation, improvement and the resistant reaction (Zou et al. 2006). They are the foremost proteolytic stomach related catalysts in specific insects and hence give supplements required to survival and fecundity. All the known individuals from the chymotrypsin family have been found in creatures. It is striking that no individual from this exceptionally fruitful family has been experienced in protozoa or plants (Rawlings and Barrett 1993). SPs are synthesized as zymogens, which require proteolysis at a particular site for initiation. Enzymatically dynamic SPs include a high specificity reactant set of three amino acid residues in their synergist area, made of histidine (His), aspartic corrosive (Asp) and serine (Ser). Biochemical and genomic examinations uncovered that chemically inert serine protease homologs (SPH) are likewise individuals from the SP family (Zou et al. 2006). SPHs have comparative successions to SPs yet need at least one of the synergist build ups. Non-proteolytic SPHs are essential segments of phenoloxidase actuation in creepy crawly inborn resistant reactions (Yu et al. 2003). Bao et al. (2014) recognized an aggregate of 90 anticipated serine protease-like genes via seeking the *N. lugens* genome succession in view of the KEGG, Swissprot and Trembl comments, which were approved utilizing the tBLASTX calculation with a cut-off E-estimation of 10^{-10}. Serine proteinases can be classified into three groups based mainly on their primary substrate preference: (i) trypsin-like, which cleave after positively charged residue; (ii) chymotrypsin-like, which cleave after large hydrophobic residues; and (iii) elastase-like, which cleave after small hydrophobic residues.

Trypsin is the main intestinal digestive serine protease enzyme responsible for the hydrolysis of the peptide bonds in which the carboxyl groups are contributed by the lysine and arginine residues. Based on the ability of protease inhibitors to inhibit the enzyme from the insect gut, this enzyme has received attention as a target for biocontrol of insect pests. The enzyme is specifically inhibited by N-α-tosyl lysine chloromethyl ketone that acts on histidine (Omondi 2005). Through the use of ester or amide

derivatives of arginine, such as N-α-tosyl arginine methyl ester (TAME) or N-α-Benzoyl-DL-arginine ethyl ester (BAEE) and N-α-Benzoyl-DL-arginine 4-nitroanilide (BApNA), digestive trypsin-like activity has been reported in most insect species (Applebaum 1985). Mostly, trypsin M_r values ranges from 20,000 to 35,000 Da and pI values are variable (range, 4–5). The pH optima is always alkaline (between 8 and 10), irrespective of the pH prevailing in midguts from which the trypsins are isolated. Nevertheless, trypsins isolated from Lepidoptera have higher pH optima that correspond to the higher pH values found in their midguts.

Isolation of inactive precursors (zymogens) of insect digestive proteinases has largely been unsuccessful (Applebaum 1985). Graf et al. (1986) suggested the occurrence of an inactive form of trypsin (trypsinogen) in midgut cells of *Aedes aegypti* (Stegomyia) based on the finding of trypsin immunoreactivity in midgut cells and on their failure to assay trypsin activity in homogenates of washed midgut cells. Nevertheless, trypsin is also immunolocalized in the glycocalyx of *Ae. aegypti* midgut cells (Graf et al. 1986), the site at which trypsin must be active and from where it cannot be removed by washing (Santos et al. 1986). The failure to assay trypsin in midgut homogenates indicates a low sensitivity of their assay procedure rather than in favour of the existence of a trypsinogen. Barillas-Mury et al. (1991) sequenced, what seemed to be a precursor of midgut trypsin in *Ae. aegypti*. Its sequence is similar to that of most trypsins, although it showed significant differences from the vertebrate trypsin precursors in the region of the activation peptide. Similar results were found with a putative trypsinogen from *Drosophila melanogaster* (Meigen) (Davis et al. 1985) and from *Simulium vittatum* (Zetterstedt) (Ramos et al. 1993). These differences suggest that the processing of precursors of insect trypsins may be different from that of vertebrates. There is evidence in *Tineola bisselliella* (Hummel) (Ward 1975) and *Bombyx mori* (Eguchi et al. 1982) that soluble trypsin is derived from membrane-bound forms. *Erinnyis ello* (Santos et al. 1986) and in *Musca domestica* (Linnaeus) (Lemos and Terra 1991a,b), trypsin is synthesized in midgut cells in an active form, but is associated with membranes of small vesicles. These vesicles then migrate to the cell apex and trypsin precursors are processed to a soluble form before being secreted. It seems that insects may control the activity of their digestive proteinases, in the absence of inactive forms, by binding the proteinases to membranes until they are released into the midgut lumen. Secretory granules isolated from the opaque zone cells from *Stomoxys calcitrans* (L.) adults contain a trypsin-like activity, which increases during incubation according to an apparent autocatalytic reaction (Moffatt and Lehane 1990). The finding by the authors that activation occurs to a different extent, if opaque zone cells are homogenized in the presence or absence of

detergent, suggests that trypsin processing in this insect is also different from that found in vertebrates.

Serine proteases comprises a catalytic triad involving three amino acid residues, His, Asp and Ser, which are essential for the catalytic process. Formation of an acyl enzyme intermediate between the substrate and the Ser amino acid is the first step during the enzyme catalysis, which proceeds through a negatively charged tetrahedral transition state intermediate thereby resulting into the cleavage of the peptide bond. Ser-hydroxyl of the enzyme is restored during the second step, during which the acyl-enzyme intermediate is hydrolysed by a water molecule to release the peptide, hence the step is called deacylation reaction. The deacylation proceeds through the reverse reaction pathway of acylation which involves the formation of a tetrahedral transition state intermediate, where a water molecule is the attacking nucleophile instead of the Ser residue. The His residue provides a general base and accepts the OH group of the active Ser (Haq and Khan 2003; Haq et al. 2004).

Cysteine Proteinases

Cysteine proteinases, endopeptidyl hydrolases with a cysteine residue in their active center are generally recognized considering the impact of their active site inhibitors (iodoacetate, iodoacetamide and E-64) and activation of the catalysts by thiol compounds (Grudkowska and Zagdańska 2004). In insect pests, the cysteine proteinases are used in the digestive processes (Rawlings and Barrett 1993), however are also found in a few different tissues, showing that they may likewise assume different other roles (Matsumoto et al. 1998). pH reliance of cysteine proteinase movement in the unrefined concentrate of insect larvae have demonstrated that this action was for the most part in the basic range (Bode and Huber 1992; Oliveira et al. 2003). The papain family contains peptidases with a wide assortment of exercises, incorporating endopeptidases with wide specificity (for example, papain), endopeptidases with exceptionally limit specificity (for example, glycyl endopeptidases), aminopeptidases, dipeptidyl-peptidase, and peptidases with both endopeptidase and exopeptidase exercises (for example, cathepsins B and H). There are likewise relatives that demonstrate no reactant action (Dubey et al. 2007). All papain-like cysteine proteases share comparative successions (Berti and Storer 1995) and have comparative 3-D structures. The auxiliary information gives solid confirmation that these proteinases all emerged from a typical precursor (Dubey et al. 2007). Proteinaceous inhibitors of cysteine proteinases are subdivided into three families (stefin, cystatin and kininogen) considering their succession homology, the nearness and

position of intrachain disulfide bonds, and the atomic mass of the protein (Turk and Bode 1991).

Cysteine proteases play an extensive variety of roles in insect pests, considering major functions in embryogenesis (Shiba et al. 2001), shedding (Liu et al. 2006), detoxification of plant protective proteins (Koo et al. 2008), insusceptible reactions (Zhang et al. 2013), and absorption (Goptar et al. 2012). The most widely contemplated part of cysteine proteases in herbivorous insect pests is their capacities as stomach related catalysts. In Coleoptera, Diptera, and Hemiptera, cysteine proteases are essential digestive enzymes (Cristofoletti et al. 2003). Numerous herbivorous insects utilize various sorts of proteases as digestive proteins. The exceptional assorted qualities and versatility of proteases expressible in the insects' nutritious tract empowers insect to safeguard themselves against an assortment of dietary poisons and antinutritional compounds they may experience in their host plants. Within the sight of protease inhibitors, insects can overproduce the current inhibitor-delicate stomach related proteases to surmount the inhibitors (Ahn et al. 2004) or increment articulation of inhibitor-inhumane protease isoforms (Bolter and Jongsma 1995; Oppert et al. 2010).

Cysteine proteinases catalyse the reaction in a similar way as serine proteinases through the formation of a covalent intermediate which involves a Cys and a His residue. The crucial Cys25 and His159 (e.g., papain) take part in the same role as Ser195 and His57, respectively, as in serine proteinases. Here the nucleophile is a thiolate ion instead of a hydroxyl group, which is stabilized through the formation of an ion pair with adjacent imidazolium group of His159. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps, without the involvement of water molecule (Kuroda et al. 2001; Yoza et al. 2002; Connors et al. 2002; Haq et al. 2004).

Aspartic Proteinases

Insects have a wide range of proteases; the larger part utilizes serine proteases as essential digestive proteases (Waniek et al. 2005), and cysteine and aspartate proteases (cathepsins B, D, H, L) as intracellular lysosomal proteins (Cho et al. 1999). In some Coleoptera and cyclorrhaphous Diptera some portion of the midgut has an acidic pH of 5.4–6.9 and 3.1–6.8, separately (Terra and Ferreira 1994). In these insects, cysteine and aspartate proteases are emitted into the lumen of the midgut as significant digestive enzymes (Padilha et al. 2009). In the triatomine *Rhodnius prolixus* Stal, as per pH judgments by means of pH markers the pH esteem substitutes sustaining conditionally in the vicinity of 5.5 and 7.4 (J.M.C. Ribeiro and

E.S. Garcia, individual correspondence). Consequently, triatomines utilize those cathepsins as stomach related proteases (Terra et al. 1996).

Aspartic proteinases do not involve a covalent tetrahedral intermediate as observed in serine and cysteine proteinases. The nucleophilic attack is attained by two concurrent proton shifts: one from a water molecule to the diad of the two carboxyl groups and a second one from the diad to the carbonyl oxygen of the protein substrate with the concomitant peptide bond cleavage. This is a general acid-base catalysis, called a "push-pull" reaction mechanism, resulting to the formation of a non-covalent neutral tetrahedral intermediate (Mares et al. 1989).

Metalloproteinases

Metalloproteases are the most varied type of catalytic proteases characterized by the requirement for a divalent metal ion for their activity (Barrett 1998). They vary extensively in their amino acid sequences and their organization, and the great majority contain a zinc atom as their catalytically active site. Some cases involve another metal atom such as cobalt, manganese or nickel. Bacterial thermolysin is a well characterized metalloproteinase, and its crystallographic structure indicates that Zn is bound by two His and one Glu amino acid residues. Most of the metalloproteinases contain the amino acid sequence HEXXH, which provides two His ligands for the Zn binding while the third ligand is either a Glu (e.g., thermolysin, neprilysin, alanyl aminopeptidase) or a His (e.g., astacin). A water molecule is also essential for the catalysis, coordinates with the metal ion as a fourth ligand in the active form of the enzyme. Other families show a distinct mode of binding with the Zn atom. About 30 families of metalloproteases have been documented, of which 17 contain only endopeptidases, 12 contain only exopeptidases and 1 (M3) contains both endopeptidases and exopeptidases. An angiotensin-converting enzyme (ACE) in insects has substantially a functional metalloprotease with a presumed role in reproduction, development and defense (Macours and Hens 2004). Endothelin-converting enzyme (ECE) is another neuropeptide degrading metalloprotease reported from insects with an endopeptidase activity (Isaac 1988).

The mechanism of action of metalloproteases is vaguely different from that of other proteases in a way that they depend on the presence of bound divalent cation. The metal ion is held in position by several amino acid residues. The catalysis involves the formation of a non-covalent tetrahedral intermediate after the attack of a Zn-bound water molecule on the carbonyl group of the scissile peptide bond. This intermediate is finally decayed by transfer of proton from the Glu to the leaving group (Skiles et al. 2004).

Conclusion

The proteinases are a noteworthy group of hydrolytic enzymes in insects and are included in digestive processes. The voracious nature of the insect pests is mainly due to the presence of several isozymes of proteolytic enzymes in their gut. Characterization of the proteolytic properties of the digestive enzymes of insect pests therefore offer an opportunity for developing suitable and effective pest management strategies via plant protease inhibitors.

Insect Adaptations to Plant PIs

Defensive mechanisms against the insect pests developed in host plants pose a substantial selection pressure on them, which have resulted in development of counter adaptations to these defenses in insects (Gatehouse 2002; Jongsma and Bolter 1997). Although plants PIs are induced in response to insect damage, many insects have adapted to plant PIs resulting in even greater loss to the plants (Steppuhn and Baldwin 2007; Parde et al. 2012). This counter defense in insect pests in response to plant PIs is a major obstacle to the management of crop protection by exploitation of PIs for a long-lasting plant defense, and thus merits an understanding of the mechanisms by which insects counteract the PI-mediated plant defense. Adaptation mechanisms adapted to PIs in insect pests has attracted the researchers to understand the mechanisms, and eventually design better approaches so that PIs can be better utilized in crop protection (Parde et al. 2012; Bolter and Jongsma 1995; Brioschi et al. 2007). Insects overcome the insecticidal effect of plant PIs either by regulating the levels of existing proteases or by synthesizing newer proteases in their gut. Thus, there could be two types of resistance or adaptation mechanisms developed in insect pests in response to protease inhibitors. In one type, insects regulate the level of proteases in their midgut that are sensitive to the plant PIs or they may have mutations in gene encoding proteases which confer resistance without losing catalytic activity or over express the protease(s) that are insensitive to plant PIs (Parde et al. 2010). These insensitive proteases are produced either constitutively and/or induced in insects to compensate the loss of inhibited protease(s) (Parde et al. 2012; Bolter and Jongsma 1995; Jongsma et al. 1995). The second mechanism involves the alternative proteases, which either compensate the loss of PI-inhibited protease(s) or degrade the plant PIs to diminish their inhibitory activity (Zhu-Salzman et al. 2008; Giri et al. 1998) (Fig. 1). Hyper-secretion of additional proteinases in response to the inhibitors requires the utilization of essential amino acid pools that could starve the insects (Broadway and Duffey 1986; Broadway 1995). In addition, hyper-production of proteases in response to ingested

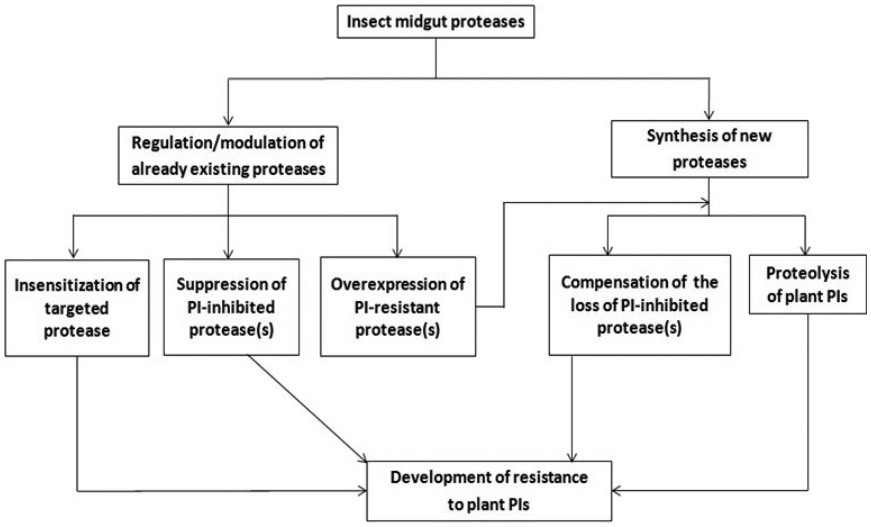

Figure 1. Adaptations of insect pests to plant PIs.

PIs leads to a further load on insect for energy and essential amino acids, resulting in delay of insect growth (Broadway and Duffy 1986). In contrast, few authors argue that the production of PI degrading proteinases derive dual benefit for insect by restoration of gut proteinase activity and the availability of valuable, sulfur-rich amino acids (Harsulkar et al. 1999).

H. armigera regulates its digestive proteinase levels against different types of PIs of *Albizia lebbeck* seeds by constitutive hyper-production of existing enzymes, trypsin, chymotrypsin and aminopeptidase activities to overcome the antinutritional effects of the inhibitor (Hivrale et al. 2013). Reduction in the serine protease activities due to ingestion of plant proteinase inhibitors is compensated with a significant induction of aminopeptidase activities in *Chilo suppressalis* and *Spodoptera exigua* (Lara et al. 2000; Vila et al. 2005). *H. armigera* larvae expressing high levels of chymotrypsin survive on a diet containing a multidomain serine PI from *Nicotiana alata* (Dunse et al. 2010). Naseri et al. (2010) demonstrated that larvae of *H. armigera* fed on soybean (cultivars L17 and Sahar) showed hyper-production of proteases in response to protease inhibition by PIs and leading to weak potential to increase its population on these cultivars. The inhibition of trypsin activity by PIs of these two soybean cultivars resulted in hyper-production of chymotrypsin-like enzymes in *H. armigera* (Naseri et al. 2010). Larvae reared on corn had the highest chymotrypsin- and elastase-like activity compared with other host plants to compensate the inhibitory effect of trypsin inhibitor of the host plant (Baghery et al. 2014). Wu et al. (1997) have reported the secretion of chymotrypsin- and elastase-like proteinases in *H. armigera* gut in response to giant taro trypsin

inhibitor. This is because due to the broader substrate specificity and significant differential interaction of chymotrypsins with the inhibitors (Peterson et al. 1995). Within different host plants, the highest general proteolytic activity was in the larvae reared on cultivars Dehghan (white kidney bean) and Arman (chickpea), indicating the presence of some PIs on these cultivars, resulting in hyper-production of proteases by midgut cells of *H. armigera* in response to protease inhibition by PIs (Hemati et al. 2012).

Proteolytic inactivation is also a significant adaptation mechanism developed in insects to resist the proteolytic inhibition by PIs, wherein they have mutations which confer greater resistance without losing catalytic activity. Trypsins insensitive to plant PIs have been characterized from *Agrotis ipsilon, Trichoplusia ni* and *H. zea* (Volpicella et al. 2003; Broadway 1997; Mazumdar-Leighton and Broadway 2001). The larvae possessed higher levels of PI-resistant digestive proteolytic enzymes when fed on artificial diet incorporated with soybean trypsin inhibitor (SBTI) (Broadway 1997). Colorado potato beetles, *Leptinotarsa decemlineata* expressed cysteine proteinases resistant to inhibitors when fed on potato leaves containing high levels of endogenous proteinase inhibitors (Bolter and Jongsma 1995). Similarly, the expression of cysteine proteinases, intestains A and C, which are insensitive to the PIs, increased in Colorado potato beetle upon feeding on potato plants with induced PIs (Gruden et al. 2004). *Heliothis virescens* expressed PI-resistant trypsin enzyme when exposed to diet containing PIs (Jongsma et al. 1995; Gatehouse et al. 1997; Bown et al. 1997). *S. exigua* has developed resistance to potato proteinase inhibitor II by induced gut proteinase activity, which is insensitive to the inhibitors (Brioschi et al. 2007; Jongsma et al. 1995). Further, *S. frugiperda* when fed on diet containing Soybean Proteinase Inhibitors (SPI), the larval gut proteases were found to be insensitive to the inhibitor (Brioschi et al. 2007; Paulillo et al. 2000). A B-type carboxypeptidase in tomato fruitworm had developed resistance to the potato carboxypeptidase inhibitor due to the rearrangement of two small regions that otherwise stabilizes the enzyme-inhibitor complex resulting into a displacement of the active-site entrance, which impairs a proper interaction between the protease and its inhibitor (Bayes et al. 2005).

The regulation of synthesis of new enzymes resistant to the inhibitors is also one of the important adaptations in insects to plant PIs. Adaptation to SPI in *S. frugiperda* involves *de novo* synthesis and up-regulation of chymotrypsin and trypsin enzymes (Brioschi et al. 2007). A new trypsin-like protease is produced in *S. frugiperda* larvae when nurtured on artificial diet incorporated with soybean PIs (Paulillo et al. 2000). Some coleopteran and lepidopteran larvae exhibited proteolytic degradation of the PIs facilitated by the insect's midgut proteinases (Giri et al. 1998; Girard et al.

1998). The diamondback moth, *Plutella xylostella* larvae have been found to be insensitive to Mustard Trypsin Inhibitor 2 (MTI2), which has been attributed to the degradation of MTI2, thus preventing the inhibitory effect of the inhibitor (Yang et al. 2009).

It has been revealed that 12 different serine proteinases were either up- or down-regulated 2- to 12-fold in *H. armigera* when fed on soybean Kunitz-type trypsin inhibitor as evidenced by gene expression studies (Gatehouse et al. 1997; Bown et al. 1997). *Callosobruchus maculatus* counteracts soybean cysteine protease inhibitors (soyacystatin N, scN) by modulating digestive enzymes and about 30 different cDNAs encoding chief digestive cathepsin L-like cysteine proteases (CmCPs) have been copied (Zhu-Salzman et al. 2003). Based on sequence similarity these CmCPs can be CmCPA and CmCPB. CmCPB was over-expressed in bruchids when fed on diet containing scN, which has higher proteolytic activity, highly effective in converting zymogens into active forms and scN into inactive form (Ahn et al. 2004, 2007). The PIs, though considered as important and highly effective defense components of plant resistance, in most of the cases, no longer serve as resistant components in plants against insect pests.

Conclusions

The coevolution between plants and insects has lead to the development of important and effective plant defense systems in plants; however, insects too have developed several strategies to avoid plant defense systems. The counter defense by insects to plant defense is highly complex and has posed a big challenge in controlling them. The studies on insect adaptation have shown that even though plants develop highly effective and dynamic defensive strategies against insect pests, these strategies are vulnerable to insect adaptation in many ways. There is a need of in-depth studies on insect adaptations to plant defense to gain an understanding of the mechanisms underlying the adaptation, and the measures that need to be taken to prevent the insects from developing such adaptations.

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