International Journal of Current Research and Academic Review

EXCELLENT PUBLISHERS

Protease inhibitors in crop protection from insects

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A B S T R A C T

World wide crop losses without the use of pesticides and other non-chemical control strategies is estimated to be about 70% of crop production, amounting to U.S. $ 400 billion. Many insect pest families are known which cause serious damage to agriculture crops. Insect pest menace is one of the major factors that destabilize crop productivity in agricultural ecosystems. They are responsible for severe reduction in crop yields, in spite of extensive use of chemical pesticides. Plant traits that are important for resistance to insect pest attack are complex and operate on many spatial scales involving direct and indirect defenses. Protease inhibitors (PIs) are one class of plant defense proteins against insect pest infestation. Plant derived protease inhibitors inactivate proteases of animals and microbial origin while rarely, inhibiting endogenous enzymes is a compelling evidence for the current view that they are involved in the protection of plants against pests and possibly pathogens. They are one of the prime candidates with highly proven inhibitory activity against insect pests and also known to improve the nutritional quality of food. Efforts are being made to explore their use in developing insect resistance in susceptible crop plants. However, a thorough understanding of insect digestive enzymes and protease inhibitors is a prerequisite to plan strategies for successful and sustainable application of PIs.

Introduction

The world wide pre-harvest losses due to insect pests despite the use of insecticides are 15% of total production representing over US $ 100 billion. The annual cost of insect control itself amounts to US $ 8 billion, thus warranting urgent economical control measures. In developing countries, the problem of competition from insect pests is further complicated with a rapid annual increase in the human population. Therefore,

in order to feed the ever expanding population, crop protection plays a vital and integral role in the modern day agricultural production to minimize yield losses. There are several families of insect pest causing damage to the crops and affecting the economy. Aphids, cabbage maggot, Colorado potato beetle, corn earworm, fall armyworm etc are common insect pests causing major losses to crops of economic

importance. No single method is likely to be adequate for all pests. Since the use of synthetic pesticides is prohibited, the organic cropping system should be focused on the prevention of pest outbreaks rather than coping with them after they occur. Successful pest management depends on the incorporation of a number of control strategies. Some strategies will target insect and disease pests separately and others will target them together. Pests in a crop do not automatically result in damage or yield loss. In some instances, low levels of insect feeding have been shown to increase crop yields. Once infestation levels reach a certain point, however, they can produce economic losses. Thresholds vary with the crop and the pest in question and must be closely monitored by the producer.

Natural insecticides

Organic certification standards prohibit the use of synthetic pesticides. The high risk of phytotoxicity should also be considered when using these products on certain plants; often the margin of error between benefit and damage to the plant is very small. In addition, there are environmental and ecological concerns surrounding some of these products. Insecticides permitted in organic agriculture include some microbial insecticides containing the bacteria *Bacillus thuringiensis*. Three main strains of these bacteria are used in insect control. One strain, marketed as Dipel or Thuricide, kills only the larvae of moths or butterflies. Another strain, marketed as Novodor, is for beetle larvae only and can be used to control Colorado potato beetles. The third strain is specifically for mosquito and fly larvae.

Though this exogenous natural pesticidal agents Bt toxin is effective in deterring plant predators, but in spite of its current successes, it may create environmental

safety and consumer health debates in future in addition to a multitude of ethical concerns [17]. The *Bacillus thuringiensis* (Bt) endotoxin gene has been successfully expressed in several crops to impart resistance against herbivorous insects. However, insects have developed resistance to Bt endotoxin by producing a proteinase(s) that inactivates the toxin or by lacking the proteinase allele required for activation of Bt protoxin. Crops resistant to insect attack offer an alternative strategy of pest control to a total reliance upon chemical pesticides. Transgenic plant technology can be a useful tool in producing resistant crops, by introducing novel resistance genes into a plant species. This technology is seen very much as forming an integral component of a crop management programme. Several different classes of plant proteins have been shown to be insecticidal towards a range of economically important insect pests from different orders; in some cases a role in the defence of specific plant species against phytophagous insects has been demonstrated.

Genes encoding insecticidal proteins have been isolated from various plant species and transferred to crops by genetic engineering. Amongst these genes are those that encode inhibitors of proteases (serine and cysteine) and a-amylase, lectins, and enzymes such as chitinases and lipoxygenases. Examples of genetically engineered crops expressing insecticidal plant proteins from different plant species, with enhanced resistance to one or more insect pests from the orders Lepidoptera, Homoptera and Coleoptera are presented. The number of different crop species expressing such genes is very diverse and ever-increasing. The viability of this approach to crop protection is considered. Production of proteinaceous inhibitors that interfere with the digestive biochemistry of insect pests is one of the naturally occurring defense mechanisms in

plants. This mechanism is manifested in the form of accumulation of one or several defense proteins such as protease inhibitors, α-amylase inhibitor, lectins and arcelins. The potential for using this natural host plant resistance in pest control across the plant genetic barriers has increased with the development of gene transfer techniques. It is therefore necessary to evaluate as many plant sources as possible for identifying the presence of proteins with ideal insecticidal properties.

It is also equally important to characterize these proteins and their encoding genes to strengthen and broaden the resistance gene pool. Protease inhibitors, α-amylase inhibitors and lectins have extensive investigations particularly in the last two decades. In addition, some related but less studied proteins like arcelins and vicilins have also invoked research attention in recent years.

The use of PIs in developing insect resistance in transgenic plants is of dual benefit, as they inhibit insect mid-gut proteinases, thereby protecting other defense proteins from proteolytic degradation. PIs block digestive proteinases in insect guts and starve them of essential amino acids. They also affect a number of vital processes, including proteolytic activation of enzymes and molting. Although plant PIs inhibit growth of insects, they do not lead to high selection pressure compared with the "wipeout" approach executed by other pest control measures (including Bt toxin).This minimizes the possibility of developing resistance in the insect population against PIs. Another merit of this approach lies in the fact that PIs are a plant's own natural defense response against phytophagous insects. PIs are present in the leaves and storage tissues, and are shown to be induced upon wounding, thereby significantly reducing the insect attack.

Plant protease inhibitors

Plant proteinase inhibitors (PIs) have been well established to play a potent defensive role against predators and pathogens. Although diverse endogenous functions for these proteins has been proposed, ranging from regulators of endogenous proteinases to act as storage proteins, evidence for many of these roles is partial, or confined to isolated examples. On the other hand, many PIs have been shown to act as defensive compounds against pests by direct assay or by expression in transgenic crop plants. The role and mechanism of action for most of these inhibitors and their respective genes are being studied in detail. These genes have been used for the construction of transgenic crop plants to be incorporated in integrated pest management programmes. Protease inhibitors (PIs) are one of the prime candidates with highly proven inhibitory activity against insect pests and also known to improve the nutritional quality of food.

The possible role of protease inhibitors (PIs) in plant protection was investigated as early as 1947, when Mickel and Standish[42] observed that the larvae of certain insects were unable to develop normally on soybean products. Subsequently the trypsin inhibitors present in soybean were shown to be toxic to the larvae of flour beetle *Tribolium confusum*[37]. The term "protease" includes both "endopeptidases" and "exopeptidases" whereas the term "proteinase" is used to describe only "endopeptidases"[48]. 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. Although, some of them may play a role in endogenous protein metabolism, most of the protease inhibitors that have been characterized from plants do not inhibit endogenous plant proteases, but have specificities for animal or microbial

enzymes[35].*In vitro* feeding trials using artificial diets containing the inhibitors have confirmed the protective role for protease inhibitors against several crop pests.

The effects of PIs on susceptible insects are generally seen as an increase in mortality, decrease in growth rate and prolongation of developmental period of the larvae. These detrimental effects are accomplished by blocking insect midgut proteinases thus impairing protein digestion, which inhibits or at least delays (in the case of weak inhibitors) the release of peptides and amino acids from dietary protein. The presence of inhibitor leads to the loss of nutrients particularly sulphur containing amino acids, and thereby weak and stunted growth and ultimate death[16].

Majority of proteinase inhibitors studied in plant kingdom originates form three main families namely leguminosae, solanaceae and gramineae[47]. These protease inhibitor genes have practical advantages over genes encoding for complex pathways i.e. by transferring single defensive gene from one plant species to another and expressing them from their own wound inducible or constitutive promoters thereby imparting resistance against insect pests[8]. This was first demonstrated by Hilder et al.[22] 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 inhibitors

Protease inhibitors (PIs) exhibit a very broad spectrum of activity including suppression of pathogenic nematodes like *Globodera tabaccum, Globodera pallida* and *Meloidogyne incognita*[54]. PIs from pearl millet inhibit growth of many pathogenic fungi including *Trichoderma reesei*[29]. These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests. Further, 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. These inhibitor families have been found specific for each of the four mechanistic classes of proteolytic enzymes and based on the active amino acids in their "reaction centre"[32], are classified as serine, cysteine, kunitz, aspartic and metallo proteases.

Serine proteinase inhibitors

Serine proteinases are not used by plants in processes involving large scale protein digestion, and hence the presence of significant quantities of inhibitors with specificity towards these enzymes in plants cannot be used for the purpose of significant quantities of inhibitors with for the purposes of regulating endogenous proteinase activity[46]. In contrast, a major role for serine PIs in animals is to block the activity of endogenous proteinases in tissues where this activity would be harmful, as in case of pancreatic trypsin inhibitors found in mammals. The serine class of proteinases such as trypsin, chymotrypsin and elastase, which belong to a common protein super family, are responsible for the initial digestion of proteins in the gut of most higher animals[15]. There are three types of digestive serine proteinases which are distinguished based on their specificity, trypsin specifically cleaving the C-terminal to residues carrying a basic side chain (Lys, Arg), chymotrypsin showing a preference

for cleaving C-terminal to residues carrying a large hydrophobic side chain (Phe, Tyr, Leu), and elastase showing a preference for cleaving C-terminal to residues carrying a small neutral side chain (Ala, Gly)[48]. Inhibitors of these serine proteinases have been described in many plant species, and are universal throughout the plant kingdom, with trypsin inhibitors being the most common type. 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 proteinase inhibitors. Such studies have provided a basic understanding of the mechanism of action[26] that applies to most serine proteinase inhibitor families and probably to the cysteine and aspartyl proteinase inhibitor families as well. All serine inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with a similar standard mechanism[36].

Serine proteinases have been identified in extracts from the digestive tracts of insects from many families, particularly those of Lepidoptera[24] and many of these enzymes are inhibited by proteinase inhibitors. The order Lepidoptera, which includes a number of crop pests, the pH optima of the guts are in the alkaline range of 9-11 where, serine proteinases and metallo-exopeptidases are most active. Additionally, serine proteinase inhibitors have anti-nutritional effect against several lepidopteran insect species[2]. Purified Bowman-Birk trypsin inhibitor[12] at 5% of the diet inhibited growth of these larvae but SBTI[34], another inhibitor of bovine trypsin, was less effective when fed at the same levels. Broadway and Duffey[11] 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 levels of 10% of the proteins in their diet. Trypsin inhibitors at 10% of the diet were toxic to larvae of the *Callosobruchus maculatus*[18] and *Manduca sexta*[49]. Other studies have also shown that proteinase inhibitors can exhibit different affinities for members of homologous proteinase families from different organisms. Three pure trypsin inhibitors, SBTI, LBI and an egg white inhibitor (EWI) inhibited trypsins and chymotrypsins from 12 animal species with wide range of variability. Recent X-ray crystallography structure of winged bean, *Psophocarpus tetragonolobus* Kunitz-type double headed alpha-chymotrysin shows 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[43]. Structural analysis of the Indian finger millet (*Eleusine coracana*) bifunctional inhibitor of alph α-amylase /trypsin with 122 amino acids has shown five disulphide bridges and a trypsin binding loop[19]. These structural analysis would greatly help in "enzyme engineering" of the native PIs to a potent form, against the target pest species than the native PIs.

Inhibitors that specifically inhibit proteolytic enzymes from microorganisms and not digestive proteases of animals are common in plant, especially legume seeds. The inhibitors of the serine class of the enzymes secreted by *Bacillus subtills* (subtilisins, or SIs) are found in sees or vegetative tissues of many legume, cereal, and tuberous crops. A powerful inhibitory activity towards was identified associated with potato inhibitor I, a potent inhibitor of chymotrypsin. Other inhibitors of subtilisin were subsequently purified from cereals and legumes. Subtilisin

inhibitors are found in *Hordeum vulgare* (barley seeds), *Vigna unguiculata* (cowpea), *Vicia faba* (broad beans), *Phaseolus vulgaris* (black beans), *Phaseolus anularis* (adzuki bean), *Vigna radiata* (mung bean), *Cicer arietinum* (chick pea) and *Canavalia ensiformis* (jack beans).

Bowman-Birk Inhibitor (BBI) family

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. These inhibitors are cysteine-rich with a molecular mass of 8-20 kDa[13].The Bowman-Birk inhibitor (BBI) and its related family of isoinhibitors comprises a closely related group of serine PIs. The protein was first identified and isolated from soybean seeds by Bowman[9] and further characterized by Birk and associates[5]. Hence the name Bowman-Birk inhibitor (BBI).These proteins are classified as double-headed serine protease inhibitors due to the presence of two reactive site domains within the same polypeptide, one each for trypsin (Lys-Ser) and chymotrypsin (Leu-ser) molecules.

The cowpea trypsin inhibitor constitutes a some-what larger gene family of four major isoinhibitors, although the exact number of active genes is not known. Three of the isoinhibitors are specific for trypsin at each active site and fourth is a trypsin chymotrypsin bifunctional inhibitor. The cowpea protease inhibitor protein is comprised of readily identifiable core region covering the invariant cysteine residues and active serine centres that are bound to highly variable amino and carboxy terminal regions.

Kunitz Proteinase Inhibitors

The kunitz inhibitors are the second major family of inhibitors which are widely distributed and often very abundant, in seeds of leguminous plants, but also occurs in other groups of plants including cereal seeds. The "typical" legume proteins are trypsin inhibitors of M_r about 21,000 with four cysteine residues that form two intra chain disulphide bonds. However, in the members of the legume sub family Mimosoideae a proteolytic cleavage occurs between the third and four cysteine residues, resulting in a heterodimeric protein comprising chains of M_r about 5,000 and 16,000 linked by a single disulphide bond. The Kunitz 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. However, not all the kunitz related proteins of legume seeds are proteinase inhibitors. The winged bean aibumin-1 storage protein from *Psophocarpus tetragonolobus* accounts for about 15% of the total seed protein. It comprises 175 amino acid reisdues with an M_r 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[33].

Cysteine Proteinase Inhibitors

Isolation of the midgut proteinases from the larvae of cowpea weevil, *C. maculatus*[31] and bruchid *Zabrotes subfaceatus* confirmed the presence of cysteine mechanistic class of proteinase inhibitors. Similar proteinases

have been isolated from midguts of the flour beetle *Tribolium castaneum*, Mexican beetle *Epilachna varivestis* and the bean weevil *Ascanthoscelides obtectus*[53]. Cysteine proteinases isolated from insect larvae are inhibited by both synthetic and naturally occurring cysteine proteinases inhibitors. The optimum activity of cysteine proteinases is usually in the pH range of 5-7, which is the pH range of the insect gut that use cysteine proteinases. Although cysteine proteinase is primarily responsible for protein digestion in *C. maculatus,* it is not clear, how the cowpea and soybean Bowman-Birk inhibitors exert their anti-nutritional effects on this organism. The rice cysteine proteinase inhibitors are the most studied of all the cysteine PIs which is proteinaceous in nature and highly heat stable[1].

Inhibitors of cysteine proteinases are now called 'cystanins' as a class and consist of at least three distinct families. 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 coleopteran 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 indicating that the proteinases is usually in the pH range of the insect gut that use cysteine proteinases. Although cysteine proteinase is primarily responsible for

protein digestion in *C. maculatus*, it is not clear how cowpea and soybean Bowman-Birk inhibitors exert their anti-nutritional effects on this organism. Advances in enzymology have revealed the existence of a variety of cysteine proteinases resulting in their classification into several families namely papain, calpin and asparagines specific processing enzyme[51]. Cystanins have also been characterized from potato, ragweed, cowpea, papaya and avocado. The rice cysteine proteinase inhibitors are the most studied of all the cysteine PIs which is proteinaceous in nature and highly heat stable. Recent three dimensional structure analysis of oryzacystatin OC-1 using NMR has showed a well defined main body consisting of amino acids from Glu 13–Asp 97 and an alpha helix with five stranded anti-parallel beta-sheet, while the N terminus (Ser 2-Val 12) and C terminus (Ala 98-Ala 102) are less defined. Further, analysis has demonstrated OC-1 to be similar to chicken cystatin which belongs to type-2 animal cystatin.

Aspartic and Metallo-Proteinase Inhibitors

Studies on aspartic proteinases in insect digestion is limited than that of cysteine proteinase. In species of six families of the order hemiptera, aspartic proteinases (cathepsin D-like proteinases) were found along with cysteine proteinases[25]. The low pH of midguts of many members of coleopteran and hemiptera provides more favourable environments for aspartic proteinases (pH optima ~3-5) than the high pH of most insect guts (pH optima ~ 8-11), where the aspartic and cysteine proteinases would not be active. The cathepsin D inhibitor (27kDa) is unusual as it inhibits trypsin and chymotrypsin as well as cathepsin D, but does not inhibit aspartyl proteases such as pepsin, rennin or cathepsin

E. The inhibitors of the metallo-carboxypeptidase from tissue of tomato and potato are polypeptides (4 kDa) that strongly and competitively inhibit a broad spectrum of carboxypeptidases from both animals and microorganisms, but not the serine carboxypeptidases from both animals and microorganisms, but not the serine carboxypeptidases from yeast and plants. The inhibitor is found in tissues of potato tubers where it accumulates during tuber development along with potato inhibitor I and II families of serine proteinase inhibitor. The inhibitor also accumulates in potato leaf tissues along with 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 disgestive enzymes i.e. trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B of higher animals and many insects[23]. Aspartic PIs have been isolated from sunflower, barley and cardoon (*Cyanara cardunculus*) flowers named as cardosin A [14].

Potato Inhibitor I and II families

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. 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 is 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 ten or more isoinhibitors of protease inhibitor I and at least three forms of inhibitor II have identified. In addition, homologues 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[30].

## Mechanism of binding enzyme and enzyme inhibitors

Knowledge on mechanisms of protease action and their regulation *in vitro* and *in vivo,* in animals, plants, microorganisms and in viruses have contributed to many practical applications for inhibitor proteins in medicine and agriculture. Most animals require proteolysis to degrade and use the component amino acids of the proteins they consume. Protease inhibitors do not pose a direct problem for humans, because foods that contains high level of these proteins are cooked, which inactivated the inhibitors. The secretion of proteases has been attributed to two mechanisms, involving either a direct effect of food components (proteins) on the midgut epithelial cells or a

hormonal effect triggered by food consumption[2]. Models for the synthesis and release of proteolytic enzymes in the midguts of insects proposed Brovosky[12] reveal that ingested food proteins trigger the synthesis and release of enzymes from the posterior midgut epithelial cells. The enzymes are released from membrane associated forms and sequestered in vesicles that are in turn associated with the cytoskeleton. The peptidases are secreted into the ectoperitrophic space between the epithelium, as a particulate complex from where the proteases move transversely into the lumen of the gut, where the food proteins are degraded.

The adverse effects of protease inhibitors in foods are more complex than simply reducing the proteolytic activities of the digestive proteases. Trypsin inhibitors in animal diets have been known for some time to evoke increased pancreatic secretions, implying that active trypsin plays a role in normal regulation of pancreatic function. This regulation by trypsin apparently involves the degradation of a 'monitor peptide' that is secreted into the gut where it regulates the release of a circulating polypeptide hormone, cholecystokinin (CCK). When CCK is released from the intestinal wall into the blood stream, it control various processes such as pancreatic secretion, gall-bladder contraction, gut mobility and appetite. Interactions of the inhibitors with trypsin and other digestive proteases, interfere with the normal degradation of the monitor peptide, which then abnormally activates the complex feedback mechanisms that produce major chronic physiological response in animals.

Thus, the presence of high levels of protease inhibitors on a continual basis can lead to chronic hyper secretion by the pancreas, loss of proteolytic activity in the gut, loss of appetite, starvation and eventually death[48].The mechanism of binding of plant protease inhibitors to the insect proteases appears to be similar with all the four classes of inhibitors. Inhibitors obeying this mechanism are highly specific and limited proteolytic substrates for their target enzymes. On the surface of each inhibitor molecule lies at least one (more in multiheaded inhibitors) peptide bond called the reactive site, which specifically interact with the active site of the cognate enzyme with a very low dissociation constant (10^7 to 10^{14} M at neutral pH values), thus, effectively blocking the active site. This peptide bond may be cleaved in the enzyme inhibitor complex but cleavage does not affect the interaction so that a hydrolysed inhibitor molecule is bound similar to an unhydrolysed one. The overall mechanism of the enzyme-inhibitor interaction, including intermediates is given below

$$\textbf{E+I} \longleftrightarrow \textbf{L} \longleftrightarrow \textbf{C} \longleftrightarrow \textbf{X} \longleftrightarrow \textbf{L*}$$
$$\textbf{E+I*}$$
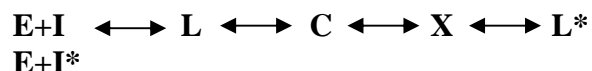
Where E is the enzyme, I and I* original and modified inhibitor, respectively, L and L* are loose, noncovalent (rapidly dissociable) complexes of E with I and I*, respectively, X is the relatively long-lived intermediate in the E+I* reaction, and C is the stable enzyme-inhibitor complex.

The secretion of protease in insect guts depends upon the midgut protein content rather than the food volume. PIs inhibit the protease activity of these enzymes and reduce the quantity of proteins that can be digested and also cause hyper-production of the digestive enzymes which enhances the loss of sulfur amino acids[49] as a result of which, the insects become weak with stunted growth and ultimately die.

The digestive proteolytic enzymes in the different orders of commercially important insect pests belong to one of the major classes of proteinases predominantly. Coleopteran and hemipteran species tend to utilize cysteine proteinases while lepidopteran, hymenopteran, orthopteran and dipteran species mainly use serine proteinases and dipteran species mainly use serine proteinases[55]. Examples from both of these classes of proteinases have been shown to be inhibited by their cognate proregions[50]. The effect of class specific inhibitors on the pest digestive enzymes is not always a simple inhibition of proteolytic activity, but recent studies have indicated the reverse may happen. It would appear that there are often two populations of digestive enzymes in target pests, those that are susceptible to inhibition and those that are resistant[10]. Some insects respond to ingestion of plant PIs such as soybean trypsin inhibitor and oryzacystatin[40] by hyper-producing inhibitor-resistant enzymes.

Regulation of proteinase inhibitors

Plant proteinase inhibitor proteins that are known to accumulate in response to wounding have been well characterized. Earlier research on tomato inhibitors has shown that the protease inhibitor initiation factor (PIIF) triggered by wounding or injury switches on the cascade of events leading to the synthesis of these inhibitor proteins and the newly synthesized PIs are primarily cytosolic[39].

The studies suggest that the production of inhibitors occurs *via* the octadecanoid (OD) pathway, which catalyzes the break down of linolenic acid and the formation of jasmonic acid (JA) to induce protease inhibitor gene expression[32]. There are four systemic signals responsible for the translocation of the wound response, which includes systemin, abscisic acid (ABA), hydraulic signals (variation potentials) and electrical signals[38]. These signal molecules are translocated form the wound site through xylem or phloem as a consequence of hydraulic dispersal. The plant systemin an 18-mer peptide has been intensely studied from wounded tomato leaves which strongly induced expression of protease inhibitor (PI) genes. Transgenic plants expressing prosystemin antisense cDNA exhibited a substantial reduction in systemic induction of PI synthesis and reduced capacity to resist insect attack. Systemin regulates the activation of over 20 defensive genes in tomato plants in response to herbivorous and pathogenic attacks. The polypeptide activates a lipid-based signal transduction pathway in which linolenic acid is released from plant membranes and converted into an oxylipin signaling molecule-jasmonic acid[48]. A wound-inducible systemin cell surface receptor with M(r) of 160 has also been identified and the receptor regulates an intracellular cascade including, depolarization of the plasma membrane and the opening of ion channels thereby, increasing the intracellular Ca^{2+} which activates a MAP kinase activity and a phopholipase A. These rapid changes play a vital role leading to the intracellular release of linolenic acid from membranes and its subsequent conversion to JA, a potent activator of defense gene transcription. The oligosaccharides, generated from the pathogen-derived pectin degrading enzymes i.e. polygalacturonase and the application of systemin as well as wounding have been shown to increase the jasmonate levels in tomato plants. Application of jasmonate or its methyl ester, methyl jasmonate, strongly induces local and systemic expression of PI genes in many plant species, suggesting that jasmonate has an ubiquitous role in the wound response. Levels of ABA have been

shown to increase in response to wounding, electrical signal, heat treatment or systemin application in parallel with PI induction[32]. Abscisic acid originally thought to be involved in the signaling pathways is now believed to weakly induce the mRNAs of wound response proteins and a concentration as high as 100 mM induced only low levels of proteinase inhibitor as compared to systemin or jasmonic acid [6] suggesting the localized role of ABA.

Protease inhibitor genes

The gene size and coding regions of the inhibitors are generally small with no introns[8] and many of these inhibitors are products of multigene families (Ryan, 1990). Bowman-Birk type double-headed protease inhibitors are assumed to have arisen by duplication of an ancestral single headed inhibitor gene and subsequently diverged into different classes i.e. trypsin/trypsin (T/T), trypsin/chymotrypsin (T/C) and trypsin/elastase (T/E) inhibitors. The mature proteins comprise a readily identifiable 'core' region, covering the invariant cysteine residues and active center serine, which are bound by highly variable amino and carboxy-terminal regions. There is a core region of 62 amino acids both between and within the different classes of inhibitor, within cowpea and with other leguminosae, including azuki bean, lima bean, mung bean and soybean. The average number of amino acid replacements in this region from all pair-wise comparisons show that the differences between the different classes of inhibitor within a species (around 16.5/62 residues) are much greater than the differences within a class between different species (around 11/62 residues). This imply that the gene duplication leading to T/T and T/C families occurred very close to the duplication, leading to the appearance of the double-headed inhibitors and that the number of silent substitutions has reached saturation in all these genes[21].

Insect resistant transgenic plants expressing PIs

A large number of protease inhibitor genes with distinct modes of action have been isolated from a wide range of crop species. Development of transgenic crop have come a long away from the first transgenic developed by Hilder and colleagues[21]. Considering the high complexity of protease inhibitor interactions in host pest systems and the diversity of proteolytic enzymes used by pests and pathogens to hydrolyze dietary proteins or to cleave peptide bonds in more specific processe[20], the choice of an appropriate proteinase inhibitor (PI) or set of PIs represents a primary determinant in the success or failure of any pest control strategy relying on protease inhibition. The choice of suitable PIs should be based on a detailed understanding of the biological system assessed. Resistant biotypes in insects may evolve after prolonged exposure to selection pressure that is mediated by an insecticidal protein or plant resistance gene.

The targeted expression of PIs in response to pest attack is another important issue. This could be controlled by using inducible promoters, such as those of PI-1185 and TobRB7 that are activated at the site of invasion by pests, pathogen and nematodes, respectively[44]. An ideal promoter should be highly responsive to invasion of the host plant by a pest or regulated by inducers just prior to pest attack. The promoter should be sufficiently active to mediate a substantial defense, specially localized to the site of pest invasion. Suitable promoters such as those regulated in response to pest invasion can be identified using promoter trapping techniques[3].

Despite these promising developments, the general usefulness of recombinant PIs in plant protection still remains to be demonstrated. The inhibitory spectrum of PIs is usually limited to proteases in one of several mechanistic classes, leaving free proteases in the surrounding medium after inhibition[4]. Due to a progressive adaptation of plant pests to the continuous occurrence of PIs in the diet, the inhibitory spectrum of protein inhibitors against the extracellular proteases of several pests is even more limited, being often restricted to the family level[52]. Non-target proteases that may allow metabolic compensation of inhibited proteolytic functions may also challenge the structural integrity of several PIs and thus potentially affect their effectiveness *in vivo*[41]. It has been observed that the presence of large amounts of inhibitors including soybean Kunitz inhibitor[10] in the diets of economical pests has made insects to adapt and produce proteases which are insensitive to the action of host plant inhibitors and the ingested PIs activate these genes. As a result, pest control using PIs in transgenic plants requires the isolation of inhibitors that are active towards these insensitive proteases[28].

Potential of Insecticidal Protease Inhibitors for Developing Transgenics Resistant to Insect Pests

Transgenic plants developed using protein inhibitors of insect digestive enzymes with a view to control crop pests are generally designed not to kill the insects that feed, but to retard their development and this is the fundamental difference between this strategy and the chemical pest control or use of Bt toxins that are aimed at complete control through pest mortality. Thus, perceived effects of the inhibitors on a pest population are usually much less dramatic than in the case with synthetic chemical pesticides.

Complete control of insects cannot be expected in any realistic trial, tending rather to increase mortality to a limited extent but to retard insect growth and development significantly. However, in an integrated pest management programme, crop protection is accomplished through the concerted effects of several complementing control measures. Moreover, the inhibitory effect of PIs could improve the efficiency of defense proteins like Bt toxins or the plants own defense proteins by preventing their degradation by the target pest proteases. Therefore, even in situations where transgene expression does not keep the pest population below the threshold for intervention, it should allow a much wider window within which intervention can be successfully employed.

The first ever transgenic plants were produced by Hilder et al [22] using cowpea trypsin inhibitor cDNA clone. The transgenic plants were resistant against herbivorous insects such *as Collosobrchus maculatus, Heliothis spodoptera* and *Diabrotica* and *Tribolium sp.* Johnson et al [27] transformed tobacco plants with gene coding tomato and potato inhibitor proteins and the transgenic plants found resistant to *M. sexta*.

Improved and extended genetic crop resistance is usually seen as a foremost possibility in the current scenario of preventing agricultural losses due to insects and diseases. Hence, another approach to slowing down insect growth is to use genes that encode for natural defensive compounds that are abundantly found in seeds. Subsequent to the preliminary observation of the role of soybean products on crop protection, the trypsin inhibitors present in soybean were shown to be toxic to the larvae of flour beetle (*Tribolium confusum*). Following these early studies, there have been many examples of protease inhibitors

active against certain insect species both *in vitro* assays against insect gut proteases and *in vivo* artificial diet bioassays. Moreover, since proteinase inhibitor genes are primary gene products, they are excellent candidates for engineering pest-resistance into plants. The availability of diverse genes from different plant sources is in itself an advantage as two or more genes can be transferred in combination (with different physiological targets). Proteinase inhibitors are also reportedly active against nematodes, viral, bacterial and fungal pathogens; thus, they may serve to have a cumulative protective effect on plants. Further, there is no evidence that proteinase inhibitors have toxic or deleterious effects on mammals. These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests.

Enzyme inhibitors are prevalent among many plant species and have been detected in many different plant organs. Plant proteins have been identified that inhibit many diverse enzymes, including animal digestive proteases and amylases; other animal enzymes, including elastase, thrombin, plasmin and kallikrein; bacterial enzymes, such as subtilisin; fungal enzymes; endogenous plant proteases and amylases; and insect digestive enzymes[47]. In plants, different roles for proteinase inhibitors have been suggested, including their action as storage proteins, as regulators of endogenous proteolytic activity as participants in many developmental processes, including programmed cell death and as components associated with the resistance of plants against insects and pathogens[45]. They may be synthesized constitutively during normal development or may be induced in response to insect and pathogen attacks. The best known antidigestive proteins in plants are proteinase inhibitors. In plants, these PIs act as anti-metabolic proteins, which interfere with the digestive process of insects. One of the important defense strategies that are found in plants to combat predators involves PIs which are in particular effective against phytophagous insects and microorganisms. The defensive capabilities of plant PIs rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development.

Future prospects of inhibitory proteins

The use of insect resistant transgenic plants is a viable means of producing crops with significantly enhanced level of resistance. Several transgenic plants expressing plant-borne inhibitor proteins have been developed in the last decade. Various approaches that are being proposed and tried by different research groups include:

(i) Gene Combinations/Packaging/ Pyramiding

The protective efficacy, spectrum of activity and the durability of resistance offered by the introduced genes can be greatly enhanced through careful design of packages of different genes that contain components which would act on quite different target insects. Protease inhibitors may have a major role in such gene pyramiding approaches. Apart from their inherent insecticidal properly, they would protect other introduced gene products form premature digestion in the insect gut and improve the overall performance through their mutually complementing or synergistic effects. The first demonstration of this approach has been the introduction of both cowpea trypsin inhibitor and pea lectin in transgenic tobacco plants where the two gene products had an additive effect on tobacco budworm caterpillars[7]. It may be a

useful approach to combine genes that encode proteinase inhibitors among themselves or along with suitable lectin, α-AI and/or B: genes so that multiple pest resistance may be achieved in a single event in agronomically important crop plants. Cross breeding of primary transformants carrying the desirable gene combinations would also prove useful in terms of enhanced insect resistance.

(ii) Protein Engineering

In-depth exploration of protein structure and function may allow researchers to use protein engineering as a strong tool for designing novel chimeric proteins for insect control. These chimeras are constructed by tailoring together the sequences that encode discrete domains of the protein intended to act on defined targets. *In vitro* mutagenesis can be exploited for creating very effective chimeric genes carrying desirable domains with defined activity spectrum. The long-term goal of protein engineering would be the constructions of modular protein that will target specific pests without any harmful effects on the beneficial organisms. In principle, any domain from any protein can be used in this modular system to construct proteins with a given set of attributes. Although still in its infancy, protein engineering will allow us to design proteins for use against the most insect pests.

(iii) Single-chain Antibodies

This approach makes use of engineering antibodies or antibody fragments specific to the target pest's essential protein and expressing it in the crop plant so that both specificity and efficacy of action can be incorporated in a single event. Besides, it has an additional advantage of avoiding action on the non-target organisms, particularly predators.

(iv) Phage Display

The technique combines *in vitro* mutagenesis, rapidity of molecular cloning, specificity of protein-protein interactions and precision of molecular screening techniques. After isolation and cloning of an ideal inhibitor gene, a large collection of its variants are prepared in the form of a library by altering its sequence at every possible position in the regions critical for its action. In fact, the gene can be modified for all the coding frames with every codon for each of the 20 possible amino acids so that the resultant changes in specificity, binding and other attributes in each of the modified product (protein) can be examined. The cloned genes are then expressed on the surface of phage particles and the displayed proteins are screened for the variant inhibitor protein, which exhibits the maximum affinity (binding) for the target protease enzyme. Thus, such technique envisages the screening of millions of cloned proteins with the desired one being physically separated from others based upon its affinity to the target larval enzyme.

Conclusion

The continuous use of pesticides for crop protection had resulted in damaging impact on biological ecosystems. The use of target specific compounds with low persistence of intrinsic plant resistance mechanisms are safer alternative strategies for effective insect pests management. Thus, insect resistant GM plants will curtail the use of those hazardous pesticides by engineering genes that encode natural biodegradable proteins with no harmful effect to animals and human beings. The availability of diverse insecticidal genes from different plant species makes it a possibility to use one or more genes in combination whose products are targeted at different

biochemical and physiological processes. The transgenic crops developed for insect resistance need to be compatible with other components of integrated pest management programmes for pest resistance to be durable and impact on agricultural systems. The use of recombinant PIs may also be an attractive way to protect plants from fungal, bacterial and viral pathogens. Biochemical screening will continue to play an important role in the search for inhibitors with desirable characteristics. Complete understanding of the structural bases of inhibitor interactions will also enable site directed mutagenesis of existing inhibitors or design of synthetic peptides to yield inhibitors specific to a small number of pests thereby, minimizing the possible environmental side-effects of the transgenic technology.

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