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(*Review paper*)

Characteristics of plant proteinase inhibitors and their applications in combating phytophagous insects

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Abstract. Plant proteinase inhibitors (PIs), which play a potent defensive role against predators and pathogens, are natural, defense-related proteins often present in seeds and induced in certain plant tissues by herbivory or wounding. This review describes the main classes of proteinase inhibitors, the distribution and localization, general properties, the main functions, and commercial applications of plant PIs. This paper also introduces the proteinase inhibitor IIs (PIN 2) and its molecular biology, including transgenic plants expressing proteinase inhibitors against insect, pests, and pathogens, esp. in lettuce (*Lactuca sativa* L.) and Chinese flowering cabbage (*Brassica campestris* ssp. *parachinensis*), which are widely cultivated or distributed in Southeast Asia, especially in South China, including Hong Kong. The morphorlogical and molecular characteristics of PIN 2-rich American black nightshade (*Solanum americanum* Mill.) was described. In addition, prospects for the application of plant PIs are also discussed.

Keywords: American black nightshade (*Solanum americanum* Mill.); Chinese flowering cabbage (*Brassica campestris* ssp. *parachinensis*); Lettuce (*Lactuca sativa* L.); Proteinase inhibitor II (PIN2); Proteinase inhibitors (PIs).

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Introduction

Proteinase inhibitors (PIs) are small proteins that are quite common in nature. They are natural, defense-related proteins often present in seeds and induced in certain plant tissues by herbivory or wounding (Koiwa et al., 1997). PIs are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms. In plants they can be counted among the defensive mechanisms displayed against phytophagous insects and microorganisms. The defensive capacities 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 (De Leo et al., 2002). Protein and peptide inhibitors of various exogenous (from invertebrates, viruses, fungi, mammals) and endogenous proteinases are widespread in seeds and may prove to be universal. Proteinase inhibitor II (PIN2), a serine proteinase inhibitor with trypsin and chymotrypsin inhibitory activities (Bryant et al., 1976), occurs in many *Solanaceae* plants, including tomato (Gustafson and Ryan, 1976), potato (Bryant et al., 1976), and tobacco (Pearce et al., 1993). PIN2 proteins could play an endogenous role in preventing uncontrolled proteolysis and/or in protecting against the foreign proteolytic enzymes of pests or pathogens (Ryan, 1989; Brzin and Kidric, 1995). Observations of their wound-inducible expression (Pena-Cortes et al., 1988; Pearce et al., 1993) have led to investigations focusing on their role in plant protection against insects (Johnson et al., 1989; Duan et al., 1996; Klopfenstein et al., 1997). Nevertheless, reports on their developmental regulation and their tissue-specific accumulation (Rosahl et al., 1986; Sanchez-Serrano et al., 1986; Hendriks et al., 1991; Pena-Cortes et al., 1991; Lorberth et al., 1992) suggest that they have endogenous functions. Recently, a different class of plant proteinase inhibitor protein, the cysteine proteinase inhibitor, was found to play a novel role in modulating programmed cell death in soybean (Solomon et al., 1999). It was discovered that both SaPIN2a and SaPIN2b are expressed in floral tissues destined to undergo developmental programmed cell death (PCD), suggesting possible endogenous roles in inhibiting trypsin- and chymotrypsin-like activities during flower development (Sin and Chye, 2004).

To enhance pest/pathogen protection in transgenic crops by the expression of plant defense proteins, two cDNA clones encoding PIN2, designated *SaPIN2a* and *SaPIN2b*, were isolated by screening an American black nightshade (*Solanum americanum* Mill.) cDNA library prepared from wounded leaves using a tomato *PIN2* cDNA (Graham et al., 1985a, 1985b; Xu et al., 2001) as a heterologous hybridization probe. *Solanum americanum* is a weed belonging to the *Solanaceae* family, which is a rich source of proteinase inhibitors (Gurusiddaiah et al., 1972; Ryan, 1973; Richardson, 1979; Brzin and Kidric, 1995). From an evolutionary viewpoint, this weed would have evolved to resist insects endemic to its growing region, and hence the *SaPIN2a* and *SaPIN2b* were further characterized (De Leo et al., 2002; Sin and Chye, 2004; Xu et al., 2004).

This article describes the main classes of proteinase inhibitors and genes used to construct transgenic plants against phytophagous insects, as well as the distribution and localization, general properties, main functions, and commercial applications of plant PIs and PIN2 and their molecular biology.

Proteolytic Enzymes

Proteolytic enzymes are intricately involved in many aspects of plant physiology and development, and their action can be divided into two different categories: limited proteolysis and unlimited proteolysis. In limited proteolysis a protease cleaves only one or a limited number of peptide bonds of a target protein leading to the activation or maturation of the formerly inactive protein e.g. conversion of prohormones to hormones. Proteases are responsible for the post-translational modification of proteins by limited proteolysis at highly specific sites. Limited proteolysis results in the maturation of enzymes, is necessary for protein assembly and subcellular targeting, and controls the activity of enzymes, regulatory proteins and peptides. In unlimited proteolysis, proteins are degraded into their amino acid constituents. The proteins to be degraded are usually first conjugated to multiple molecules of the polypeptide ubiquitin. This modification marks them for rapid hydrolysis by the proteasome in the presence of ATP. Another pathway consists of the compartmentation of proteases e.g. in lysosomes. Proteins transferred into this compartment undergo a rapid degradation. Proteolytic enzymes are necessary for protein turnover. Degradation of damaged, misfolded, and potentially harmful proteins provides free amino acids required for the synthesis of new proteins. Furthermore, the selective breakdown of regulatory proteins by the ubiquitin/proteasome pathway controls key aspects of plant growth, development, and defense. Proteases are clearly involved in all aspects of the plant life cycle ranging from the mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs (Loukas, 2002; Creemers, 2002; Schaller, 2004).

Protease, Proteinase or Peptidase?

Several almost-overlapping terms are current for the group of enzymes that hydrolyze peptide bonds. These names include peptidases, peptide hydrolase, proteases, proteinases, and proteolytic enzymes (Barrett et al., 1998), which originally had slightly different meanings and are now nearly synonymous (Barrett, 1999). The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, 1992) recommended the term peptidase as the general term for all enzymes that hydrolyze peptide bonds. These are then subdivided into exopeptidases, which cleave one or a few amino acids from the N- or C-terminus, and endopeptidases, which cleave the internal peptide bonds of polypeptides. To keep consistent with current usage of the literature in related fields, the nomenclature suggested

by Barrett (1986) is adopted in this paper. The term "protease" will encompass both exopeptidases and endopeptidases while "proteinase" will describe only endopeptidases. In the PLANT-PIs, a database for protease inhibitors and their genes in higher plants, "protease" is adopted as a formal word (De Leo et al., 2002).

Proteases are widely found in plants and animals as well as in microorganisms (Kenny, 1999). Based on an analysis of the complete sequences of several genomes, it is estimated that about 2% of all gene products are proteases (Barrett et al., 1998). About 500 well-documented proteases (peptidases) are included in the recently published Handbook of Proteolytic Enzymes (Barrett et al., 1998). Proteases play crucial roles in the physiology and pathology of living organisms by controlling the synthesis, turnover, and function of proteins (Turk, 1999). Figure 1 lists the main classes of proteases defined in the Enzyme Nomenclature of the IUBMB (NC-IUBMB, 1992). The classification of exopeptidases is based on their actions on substrates while the endopeptidases are divided by their active sites.

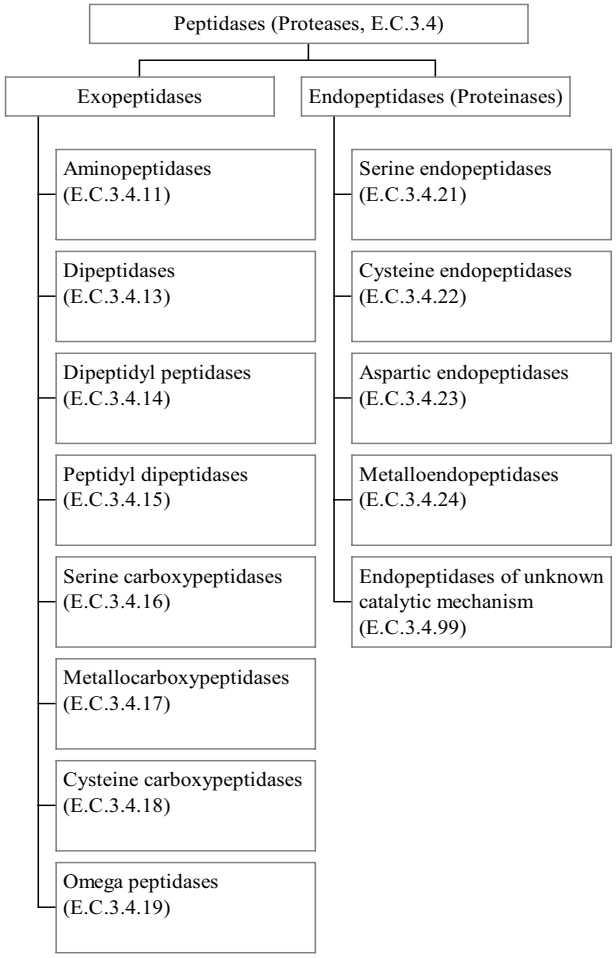

Figure 1. The main classes of proteases (peptidases) according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, 1992). NC-IUBMB recommended the term peptidase as the general term for all enzymes that hydrolyze peptide bonds. This is subdivided into exopeptidases cleaving one or a few amino acids from the N- or C-terminus, and endopeptidases cleaving internal peptide bonds of polypeptides. The classification of exopeptidases is based on their actions on substrates while the endopeptidases are divided by their active sites. Proteinases are divided into four groups: the serine proteinases, the cysteine proteinases, the aspartic proteinases, and the metalloproteinases.

Classification of Proteinase

Proteinases are classified according to their catalytic mechanisms. Four mechanistic classes have been recognized by IUBMB: the serine proteinases, the cysteine proteinases, the aspartic proteinases, and the metalloproteinases. This classification by catalytic types has been suggested to be extended by a classification by families based on the evolutionary relationships of proteases (Rawlings and Barrett, 1993). This classification is available in the SwissProt database.

In addition to these four mechanistic classes, another section of the Enzyme nomenclature is allocated for proteases of unidentified catalytic mechanism. This indicates that the catalytic mechanism has not been identified, but the possibility remains that novel types of proteases do exist.

The Serine Proteinases

This class comprises two distinct families. The chymotrypsin family, which includes the mammalian enzymes such as chymotrypsin, trypsin, or elastase or kallikrein, and the substilisin family, which includes the bacterial enzymes like subtilisin. Though the general 3D structure is different in the two families, they have the same active site geometry, and catalysis proceeds via the same mechanism. The serine proteinases exhibit different substrate specificities, which are related to amino acid substitutions in the various enzyme subsites interacting with the substrate residues. Some enzymes have an extended interaction site with the substrate. Others have a specificity restricted to the P1 substrate residue (Schaller, 2004).

Three residues which form the catalytic triad are essential in the catalytic process, i.e. His 57, Asp 102, and Ser 195 (chymotrypsinogen numbering). The first step in the catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential Serine. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate, and then the peptide bond is cleaved. During the second step, or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme. The deacylation, which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. 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 reactive Ser (Haq and Khan, 2003; Haq et al., 2004).

The Cysteine Proteinases

This family includes the plant proteases—such as papain, actinidin or bromelain—several mammalian lysosomal cathepsins, and the cytosolic calpains (calcium-activated) as well as several parasitic proteases (e.g. Trypanosoma, Schistosoma). Papain is the archetype and the best studied member of the family. Recent elucidation of the X-ray structure of the Interleukin-1-beta Converting Enzyme has revealed a novel type of fold for cysteine proteinases. Like the serine proteinases, catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The essential Cys 25 and His159 (papain numbering) play the same role as Ser 195 and His 57, respectively. The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighboring imidazolium group of His159. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps, and then a water molecule is not required (Kuroda et al., 2001; Yoza et al., 2002; Connors et al., 2002; Haq et al., 2004).

The Aspartic Proteinases

Most of aspartic proteinases belong to the pepsin family. This family includes digestive enzymes like pepsin and chymosin, lysosomal cathepsins D, processing enzymes like renin, and certain fungal proteases (penicillopepsin, rhizopuspepsin, endothiapepsin). A second family comprises viral proteinases, such as the protease from the AIDS virus (HIV), also called retropepsin. Crystallographic studies have revealed these enzymes to be bilobed molecules with the active site located between two homologous lobes. Each lobe contributes one aspartate residue of the catalytically active diad of aspartates. These two aspartyl residues are in close geometric proximity in the active molecule, and one aspartate is ionized while the second one is unionized at the optimum pH range of 2-3. Retropepsins, are monomeric, i.e. carry only one catalytic aspartate, and then dimerization is required to form an active enzyme (Mares et al., 1989).

In contrast to serine and cysteine proteases, catalysis by aspartic proteinases do not involve a covalent intermediate though a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfers: 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 substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a “push-pull” mechanism, leads to the formation of a non-covalent neutral tetrahedral intermediate (Mares et al., 1989).

The Metallo-Proteinases

The metallo-proteinases may be one of the older classes of proteinases and are found in bacteria and fungi as well as in higher organisms. They differ widely in their sequences and their structures, but the great majority contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of the activity. Bacterial thermolysin has been well characterized, and its crystallographic structure indicates that zinc is bound by two histidines and one glutamic acid. Many enzymes contain the sequence HEXXH, which provides two histidine ligands for the zinc while the third ligand is either a glutamic acid (thermolysin, neprilysin, alanyl aminopeptidase) or a histidine (astacin). Other families exhibit a distinct mode of binding the Zn atom. The catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group (Skiles et al., 2004).

Protease Inhibitors

The protease inhibitor (PI) proteins, the natural antagonists of protease, are small proteins which are quite common in nature and also present in all life forms (Fritz, 2000). The corresponding inhibitors of most proteases occur in nature (Fritz, 2000). Most PIs interact with their target proteases by contact with the active (catalytic) site of the protease, resulting in the formation of a stable protease-inhibitor complex that is incapable of enzymatic activity (Norton, 1991). Protease inhibitors have an enormous diversity of function by regulating the proteolytic activity of their target proteinases (Leung et al., 2000). Proteinases can be either reversible or irreversible. Reversible proteinases react in the absence or above critical concentrations of their inhibitors. Often in the literature, cocktails of inhibitors are made up, and little thought goes into what they are effective against, what concentrations are needed, and how long they are stable in an aqueous environment. A case in point is PMSF, a widely used serine proteinase. This has a half-life in water of 15-60 min (depending on your reference source), so it should be added just before the cell lysis. PMSF is not very soluble in water and should be kept at -20°C in dry methanol/propanol (Konarev et al., 2004; Sin and Chye, 2004; Xu et al., 2004).

In the absence of information about which class of proteinase(s) may be degrading a particular sample, a proteinase cocktail can be obtained from the following individual stocks (Table 1).

Other proteinase inhibitors that may be considered include aprotinin (inhibits serine proteinases including trypsin, chymotrypsin, plasmin, trypsinogen, urokinase, kallikrein, and human leukocyte, but not pancreatic elastase- use at 100-500 μm); leupeptin (inhibits papain, calpain, trypsin, cathepsin B - use at 1-5 mM); AEBSF (inhibits serine proteinases including trypsin and chymotrypsin - use at 10-100 mM); and bestatin (inhibits aminopeptidases including leucine aminopeptidase and alanyl aminopeptidase-use at 1-5 mM). Proteinase inhibitors dissolved in DMSO that will be added to live cells should be prepared so that the final DMSO concentration is less than 0.5% (Konarev et al., 2004; Sin and Chye, 2004; Xu et al., 2004).

Table 1. Stock and working concentrations of proteinases and their inhibitors.

Proteinase class	Inhibitor	Stock concentration	Working concentration
Serine proteinases	PMSF	100 mM in methanol	1 mM
Metallo proteinases	EDTA	500 mM in H_2O	1 mM
Serine proteinases	Benzamidine	100 mM in H_2O	1 mM
Acid proteinases	Pepstatin A (inhibits renin, cathepsi D, chymosin, proteinase B)	1 mg/ml in H_2O	1 µg/ml
Thiol proteinases (serine & cysteine proteinases)	Leupeptin	1 mg/ml in H_2O	1 µg/ml

Table 2 lists families of protease inhibitors from all biological sources (modified from Reeck et al., 1997). They are classified by sequence similarity and named after a prominent member within the family, e.g. PIN2 belongs to the Potato inhibitor II family (No. 6 in Table 2).

It is worth noting that the properties of many PIs are deduced from their cDNA-derived amino acid sequences. Hence the classifications based on sequence similarity do not entirely correspond to the inhibitory properties, which are established by activity assay with proteins. For example, a potato proteinase inhibitor II (PPI II) that is a member of the potato inhibitor II family exhibits predominantly chymotrypsin inhibitor activity while other members of this family have trypsin and chymotrypsin inhibitor activity (McManus et al., 1994b). In contrast, a pumpkin proteinase inhibitor (CMTI-V) that belongs to the potato inhibitor I family is a trypsin inhibitor, unlike other members that are chymotrypsin inhibitors (Krishnamoorthi et al., 1990).

Proteinase Inhibitors (PIs) in Plants

Serine proteinase inhibitors are universal throughout the plant kingdom and have been described in many plant species. Therefore, the number of known and partially characterized inhibitors of serine proteinases is enormous (Haq et al., 2004). Serine proteinase inhibitors have been reported from a variety of plant sources and are the most-studied class of proteinase inhibitors (Mello et al., 2002; Haq and Khan, 2003). Chiche et al. (2004) first introduced the squash inhibitor, a well-established family of highly potent canonical serine proteinase inhibitors isolated from Cucurbitaceae. The squash inhibitors were among the first discovered proteins with the typical knottin fold shared by numerous peptides extracted from plants, animals, and fungi.

Plant cystatins or phytocystatins are the second most-studied class of inhibitors and have been identified and characterized from several plants, viz., cowpea, potato,

Table 2. Protein families that contain proteinase inhibitors (Based on Reeck et al., 1997).

Family name	Approximate domain size (residues)	Number of domains
1. Bovine pancreatic trypsin inhibitor	60	1
2. Kazal serine protease inhibitor	55	up to 7
3. Kunitz soybean trypsin inhibitor	180	1
4. Bowman-Birk inhibitor	35	2
5. Potato inhibitor I	70	1
6. Potato inhibitor II	50	1 or 2; up to 8 in original translation product
7. Squash inhibitor	30	1
8. Barley trypsin inhibitor	120	1
9. Thaumatin	200	1
10. *Ascaris* trypsin inhibitor	60	1
11. Locust inhibitor	35	1; 2 in original translation product
12. Ecotin	140	2 subunits
13. Serpin	400	1
14. Streptomyces subtilisin inhibitor	110	1
15. Hirudin	65	1
16. Cystatin	110	up to 8
17. Calpastatin	140	4
18. Potato carboxypeptidase inhibitor	40	1
19. *Ascaris* carboxypeptidase inhibitor	40	1
20. Collagenase inhibitor	200	1
21. *Ascaris* pepsin inhibitor	150	1
22. α-2-Macroglobulin	1500	2 or 4 subunits

cabbage, ragweed, carrot, papaya, apple fruit, avocado, chestnut, and Job's tears. Cystatins have also been isolated from seeds of a wide range of crop plants. These crop plants include those of sunflower, rice, wheat, maize, soybean, and sugarcane (Kuroda et al., 2001; Yoza et al., 2002; Connors et al., 2002; Haq et al., 2004).

Aspartic proteinase inhibitors are a relatively less-studied class partly due to their rarity, and the metallo-proteinase inhibitors in plants are represented by the metallo-carboxypeptidase inhibitor family in tomato and potato plants (Hass et al., 1975; Graham and Ryan, 1981).

The Distribution and Localization of Plant PIs

Some twelve families of inhibitors can be recognized based on their amino acid sequences and target proteinases (Shewry, 1999). However, most studies have been carried out on crop plants (cereals, legumes, and solanaceous species) with economically unimportant species being comparatively neglected (Konarev et al., 2004). Soybean trypsin inhibitor was the first PI isolated and characterized. Since then many PIs have been found widely distributed throughout the plant kingdom. Most of plant PIs that have been characterized are from the Gramineae (Poaceae), Leguminosae (Fabaceae), and *Solanaceae* families (Brzin and Kidric, 1995). PLANT-PIs is a database developed to facilitate retrieval of information on the distribution and functional properties of protease inhibitors (PIs) in higher plants. Currently PLANT-PIs contains information for 495 inhibitors (plus several isoinhibitors) identified in 129 different plants (De Leo et al., 2002). PIs are usually found in storage organs, such as seeds and tubers, but their occurrence in the aerial part of plants, as a consequence of several stimuli has also been widely documented (De Leo et al., 2002). PIs may accumulate to about 1 to 10% of the total proteins in these storage tissues. An increasing number of PIs is found in non-storage tissues, such as leaves, flowers and roots (Brzin and Kidric, 1995; Xu et al., 2001; Sin and Chye, 2004). Some PIs also occur in yeast (Matern et al., 1979) and other fungi (Richardson, 1977).

As for the intracellular localization, only several plant PIs have been investigated. A trypsin inhibitor was found localized in the cytosol of mung bean cotyledonary cells (Chrispeels and Baumgartner, 1978). Soybean trypsin inhibitor (SBTI) is mainly present in the cell walls, with lesser amounts in protein bodies, the cytoplasm, and the nuclei of cotyledonary and embryonic cells. Soybean Bowman-Brik inhibitor (SBBI) occurs in protein bodies, the nuclei, and to a lesser extent the cytoplasm. In contrast to SBTI, some SBBI has been located in the intercellular space but not in the cell wall (Horisberger and Tacchini-Vonlanthen, 1983). The wound-induced inhibitors accumulate in vacuoles of tomato, wild tomato, and potato leaves. Xe et al. (2004) described the expression of a PIN2 protein from *S. americanum* Mill. in phloem of stems, roots, and leaves, suggesting a novel endogenous role for PIN2 in phloem. Further research showed that both SaPIN2a and SaPIN2b are expressed in floral tissues (Sin and Chye, 2004). In general, the exact subcellular location of many of the PIs is still unknown (Richardson, 1977; Norton, 1991). Therefore, further research is needed to illustrate their exact subcellular location.

General Properties of Plant PIs

Generally speaking, plant PIs vary from 4 to 85 kDa, with the majority in the range of 8 to 20 kDa (Hung et al., 2003). Plant PIs usually have a high content of cysteine residues (Richardson, 1991) that form disulfide bridges (Greenblatt et al., 1989; Hung et al., 2003) and confer resistance to heat, extremes in pH, and proteolysis (Richardson, 1991). For example, a trypsin inhibitor (BCTI) with molecular weight of 8 kDa was purified from seeds of *Brassica campestris*. The BCTI was found to be a thermostable Bowman-Birk type TI that inhibits trypsin at the molar ratio 1:1. The stability of BCTI is apparently related to the presence of the disulfide bridge (Hung et al., 2003).

Studies on the biosynthesis of several plant PIs demonstrated these PIs are synthesized as either prepro-proteins (Graham et al., 1985a) or pre-proteins (Graham et al., 1985b) that are processed in vivo either during or after synthesis to produce the native PIs (Nelson and Ryan, 1980). Some small PIs are derived in vivo from the post-translational processing of multidomain precursors (Sanchez-Serrano et al., 1986; McManus et al., 1994a; Miller et al., 2000). Many PIs are produced in response to various stress conditions, e.g. pathogens, insects, wounding, and environmental stresses such as salt (Koiwa et al., 1997).

A common opinion is that most known plant PIs do not inhibit endogenous plant proteases but have specificities for animal or microbial enzymes (Sanchez-Serrano et al., 1986). These observations may result from the fact that most studies used commercially available proteases, e.g. trypsin, chymotrypsin, elastase, and subtilisin from animal or microbial sources such as the test enzyme in the activity assays (Brzin and Kidric, 1995). However none of these test enzymes are likely to be the true physiological target enzymes for most of the characterized plant PIs.

The Main Functions of Plant PIs

The main function of plant PIs are thought to be in plant defense and the regulation of endogenous proteinases, but they may also function as storage proteins (Mosolov et al., 2001; Birk, 2003; Shewry, 2003). The possible role of PIs in plant protection was envisaged as early as 1947 when Mickel and Standish observed that the larvae of certain insects were unable to develop normally on soybean products (Haq et al., 2004). They are of interest as potential sources of resistance against pests and pathogens in transgenic plants and as drugs with antiviral and other properties as well as providing markers for studies of plant diversity and evolution (Konarev et al., 2002; Lawrence and Koundal, 2002; Korsinczky et al., 2004). It is well established that PIs protect plants from insects and other pathogens. The defensive role of PIs is based on their inhibitory activities towards the digestive enzymes of insect and other pathogens' proteases involved in some vital processess, resulting either in a critical shortage of essen-

tial amino acids (Hilder et al., 1993; Jongsma and Bolter, 1997) or interfering with important biochemical or physiological processes of insects and other pathogens, such as the proteolytic activation of enzymes, molting of insects, or replication of viruses (Gutierrez-Campos et al., 1999). The activity of PIs is due to their capacity to form stable complexes with target proteases, blocking, altering or preventing access to the enzyme active site. PIs active toward serine proteases, the most widespread in nature, act as a potential substrate for proteases. Residues forming the scissile peptide bond are indicated as P1-P1' and are generally located on an external loop of the protein, interacting with proteases. The P1 residue determines the specific type of serine protease inhibited. Other residues around the reactive site also play a role in determining the strength of the PI-enzyme interaction (De Leo et al., 2002).

Support for a defensive role of plant PIs initially came from studies of insects raised on artificial diets containing PIs and in vitro inhibition assays of insect gut proteases with purified PIs from various plant sources. The results of these studies strongly implicate plant PIs in interference with the growth and development of many phytophagous insects (Reeck et al., 1997). The first convincing evidence that PIs are part of the natural defensive chemicals of plants was the demonstration that wounding of tomato and potato leaves by Colorado potato beetles induced a rapid accumulation of proteinase inhibitor I (PIN1), not only in the damaged leaves, but also in distal, undamaged leaves (Green and Ryan, 1972). The correlation between the levels of PIs present in seeds of various cowpea varieties and the resistance to a major insect pest (*Callosobruchus maculatus*) also indicated a protective role for PIs in crops (Gatehouse et al., 1979).

The direct evidence for the involvement of PIs in the plant defense system has come from studies on transgenic plants. As their role as inhibitors is simply achieved by the activation of single genes, several transgenic plants expressing PIs have been produced in the last two decades and tested for enhanced defensive capacities, with particular efforts against pest insects. Ever since, an ever more complex scenario about the interaction between insect proteases and plant PIs has been emerging (De Leo et al., 2002). A cowpea protease inhibitor (CpTI) was shown for the first time to confer resistance to feeding by the tobacco budworm (*Heliothis virescens*) when the CpTI gene was expressed in transgenic tobacco (Hilder et al., 1987). Since then, many insect-resistant transgenic plants have been generated (Table 2).

The protective role of wound-induced PIs in tomato plants was further demonstrated by the elegant work on genetically engineered genes encoding components of the inducible systemic signaling system. Orozco-Cardenas and co-workers (1993) showed that the inability of transgenic tomato plants to produce PIN1 and PIN2—caused by constitutively expressing an antisense gene of prosystemin, the precursor protein of systemin, a powerful plant PI inducer—reduced resistance towards *Manduca sexta larvae*. On the other hand, Royo and co-workers (1999) demonstrated that antisense-mediated depletion of a potato lipoxygenase, a key enzyme in the biosysthesis of the wound-signaling molecule jasmonic acid, reduced wound induction of PIs and increased weight gain of insect pests.

In addition to insect pests, PIs also have great potential in generating transgenic plants with enhanced resistance to other pathogens, e.g. nematodes, fungi, bacteria, and viruses, the survival and/or invasion of which require proteolytic activities. Plant proteinase inhibitors are known to confer natural as well as engineered protection against nematode attack (Atkinson et al., 2003; Cai et al., 2003; McPherson and Harrison, 2001). Nematode control with PIs expressed in transgenic tomato (Urwin et al., 1995), *Arabidopsis thaliana* (Urwin et al., 2000), and rice (Vain et al., 1998) has been well demonstrated, and the technology has been patented (Hepher and Atkinson, 1992). Transgenic tobacco plants expressing rice cysteine proteinase inhibitor showed an enhanced resistance against potyviruses (Gutierrez-Campos et al., 1999). Proteinase inhibitors have also been implicated to play a role in the plant's natural defense towards fungal infections (Soares-Costa et al., 2002); Trypsin inhibitors from buckwheat seeds (Dunaevskii et al., 1994) and trypsin and chymotrypsin inhibitors from cabbage foliage (Lorito et al., 1994) have been shown to have antifungal activities.

Since plants differ in the array of PIs they can display, just as insects differ in their protease content, every plant defence strategy based on the use of transgenic PIs must be realised on the basis of a deep case-by-case investigation. The capacity of some insects to up-regulate the expression of insensitive proteases, when fed on dietary PIs, has also been reported. Unfortunately, studies on the structure and activity of single insensitive proteases, that would allow us to better understand the nature of their resistance, have not yet been reported (De Leo et al., 2002).

The protective functions of PIs have initiated many studies on plant PIs. Few reports concerning the endogenous role of PIs in plant (Brzin and Kidric, 1995) have appeared. Hou and Lin (2002) pointed out that the storage proteins of sweet potato (*Ipomoea batatas* [L.] Lam) trypsin inhibitor (SPTI) inhibit one endogenous serine protease (Arg-1), and sweet potato TIs were hydrolyzed by the same proteinase in vitro. The activity of Arg-1 was completely inhibited by SPTI in a dose-dependent manner (Hou and Lin, 2002; Hou et al., 2000; 2002; Huang et al., 2004). The most likely physiological function of PIs in plant is to regulate cell proteolysis by inhibiting endogenous proteases and hence control protein turnover and metabolism (Richardson, 1977; Ryan, 1989). PIs in storage organs, e.g. seeds and tubers, apart from being part of a pre-infection defense system, may merely serve as reserve proteins that are mobilized during germination and sprouting (Norton, 1991).

The PIs known to play an endogenous role are those found in seeds (Brzin and Kidric, 1995). A number of labs have reported that some seed PIs are active towards endogenous seed proteases (Richardson, 1977; Brzin and

Kidric, 1995). It is reasonable to assume that in dormant seeds these proteases are kept inactive by the presence of PIs. The decline of PI content during germination correlated with increased activation of these proteases in the mobilization of storage proteins (Richardson, 1977).

The physiological functions of many plant PIs are largely inferred from their developmental and tissue-specific expression patterns (Habu et al., 1996; Clark et al., 1997) and/or the expression patterns in response to exogenous application of various growth regulators (Jacobsen and Olszewski, 1996). However, many physiological functions of plant PIs under normal conditions remain to be elucidated.

Serine proteinase inhibitors are widespread in the plant kingdom. Their physiological roles include the regulation of endogenous proteinases during seed dormancy, reserve protein mobilization, and protection against the proteolytic enzymes of parasites and insects. Moreover, they may also act as storage or reserve proteins (Haq et al., 2004). To allow secretion of the Cry1Ac protein into the intercellular space, the signal peptide sequence of potato proteinase inhibitor II (pinII) was N-terminally fused to the Cry1Ac encoding region. The results showed that pinII signal peptide sequence enhanced the expression of Cry1Ac protein and led to the secretion of the Cry1Ac protein in transgenic tobacco plants (Liu et al., 2004).

SaPIN2a, a proteinase inhibitor II from *S. americanum* Mill. is highly expressed in the phloem and could be involved in regulating proteolysis in the sieve elements (Xu et al., 2001). The discovery that heterogeneously expressed SaPIN2a in transgenic lettuce inhibits plant endogenous protease activity further indicates that SaPIN2a regulates proteolysis and could be potentially exploited for the protection of foreign protein production in transgenic plants (Xu et al., 2004). A novel role for proteinase inhibitor genes as modulators of PCD in plants has been proposed by Solomon et al. (1999). They have demonstrated that the ectopic expression of cystatin genes inhibited the induced cysteine protease activity and blocked PCD triggered indirectly by an avirulent strain of *Pseudomonas syringae* pv *glycinea* or directly by oxidative stress. Sin and Chye (2004) found that both SaPIN2a and SaPIN2b are expressed in floral tissues destined to undergo developmental PCD, suggesting possible endogenous roles in inhibiting trypsin- and chymotrypsin-like activities during flower development.

Commercial Applications of Plant PIs

As stated above, plant PIs are involved in plant defense, regulation of endogenous proteinases, and protein storage. Whether plant PIs can be used in commerce has drawn great attention, and by 1991, plant PIs had already appeared in therapeutic use and laboratory applications (Richardson, 1991; Birk, 1993; Troll and Kennedy, 1993; Banerji and Fernandes, 1994; Abdel-Meguid et al., 2000; Leung et al., 2000; Mendes-Silva et al., 2003; Neuhof et al., 2003; Park and Ohba, 2004; Park et al., 2004). A great deal of early work on the therapeutic possibilities of plant PIs in the treatment of a wide range of disorders, such as pancreatitis, shock, allergy and inflammation associated with enhanced proteolytic activities, had resulted in several kallikrein trypsin inhibitor-based drugs (Richardson, 1977). Epidemiological studies of the decreased occurrence of breast, colon, and prostatic cancers in vegetarian populations suggested the role of plant PIs in preventing these cancers (Birk, 1993). This observation has led to extensive studies of plant PIs as cancer chemopreventive agents (Troll and Kennedy, 1993). Plant PIs active towards proteases that regulate human physiological processess, e.g. cell signaling/migration, digestion, fertilization, growth, differentiation, immunological defense, wound healing and apoptosis, have great potential in therapeutic applications (Abdel-Meguid, 2000; Leung et al., 2000).

Several plant PIs such as soybean trypsin inhibitor, which are readily available from commercial sources or conveniently prepared in relatively large quantities at low cost, have been successfully used for the affinity purification of their inhibited proteases from a wide variety of sources (Richardson, 1977; 1991).

Proteinase Inhibitor IIs and their Molecular Biology

PIs of the potato inhibitor II family (PIN2) are the best characterized plant serine PIs in terms of their molecular properties (Bryant et al., 1976; Richardson, 1979; Xu et al., 2001; Xu et al., 2004; Sin and Chye, 2004). Although PIN2s have been found in various tissues, e.g. tubers (Bryant et al., 1976), fruits (Richardson, 1979), wounded leaves (Pearce et al., 1993), and flowers (Sin and Chye, 2004), the presence of PIN2 in seeds has not been reported (Richardson, 1991).

Potato PIN2 is the first characterized PIN2 protein purified from potato tubers (Bryant et al., 1976) while the first complete PIN2 amino acid sequence determined was that from exocarps of *S. melongena* L. (Richardson, 1979). The nucleotide sequences of tomato PIN2 cDNA (Graham et al., 1985a) and potato PIN2 gene (Keil et al., 1986) were subsequently obtained. More and more PIN2 cDNA or gene sequences can be found from GenBank.

PIN2s are encoded by a small multigene family based on Southern blot analysis (Rosahl et al., 1986; Balandin et al., 1995). An intron is located within the coding region of the N-terminal signal peptide, and the relative position of this intron is highly conserved among the genes from potato and tobacco (Balandin et al., 1995). Almost all PIN2 amino acid sequences deduced from their cDNA sequences contain a highly hydrophobic N-terminal region of 24-31 residues that is expected to function as a signal peptide for subcellular targeting (Sanchez-Serrano et al., 1986; Balandin et al., 1995; Choi et al., 2000). The first two well-characterized PIN2s from tomato (Graham et al., 1985a, 1985b) and potato (Sanchez-Serrano et al., 1986) have two domains with trypsin and chymotrypsin reactive sites, respectively. Since then, a lot of reports have PIN2 containing up to eight domains. These domains, no matter how many in one particular PIN2, are homologous and may have

resulted from gene duplicated-elongation events (Balandin et al., 1995). Each PIN2 domain contains eight highly conserved cysteine residues (Balandin et al., 1995; Choi et al., 2000) that are involved in disulfide bonding (Lee et al., 1999).

It is well-known that some genes are expressed constitutively, and some are developmentally expressed. As for plant PIs, the best-known property of PIN2s is their wound-inducible expression (Graham et al., 1986; Pena-Cortes et al., 1988). The expression of PIN2s is also induced by pathogen-related stresses, e.g. fungal elicitor (Rickauer et al., 1989), bacterial infection (Pautot et al., 1991), and viroid infection (Gadea et al., 1996); plant hormones, e.g. abscisic acid (Pena-Cortes, et al., 1988), auxin (Taylor et al., 1993) and ethylene (O'Donnell et al., 1996); signal molecules, e.g. oligogalacturonide fragments (Bishop et al., 1984), systemin (Pearce et al., 1991) and jasmonic acid (Farmer et al., 1992); and sucrose (Johnson and Ryan, 1990). In addition, PIN2 expression induced by wounding, jasmonic acid, and systemin is inhibited by aspirin and salicylic acid (Doherty et al., 1988; Pena-Cortes et al., 1993; Doares et al., 1995).

PIN2 that usually possesses reactive sites towards both trypsin and chymotrypsin (Gatehouse, 1999; Xu et al., 2001; Xu et al., 2004; Sin and Chye, 2004) has a great potential in crop protection because the major digestive endoproteases in the insect gut are the serine proteases trypsin and chymotrypsin. The protective functions of PIN2s from tomato (Johnson et al., 1989), potato (Klopfenstein et al., 1997), and ornamental tobacco (Charity et al., 1999) have been demonstrated by the enhanced insect resistance of transgenic plants expressing these PIN2s.

The heterologous expression of serine proteinase inhibitor II (PIN2) proteins confers insect resistance in transgenic plants. Xu et al. (2001) have cloned two cDNAs encoding *S. americanum* PIN2 proteins, SaPIN2a and SaPIN2b. SaPIN2a is highly expressed in stem, particularly in the phloem, suggesting it could possibly regulate proteolysis in the sieve elements. When SaPIN2a was expressed in transgenic lettuce, an inhibition of endogenous trypsin- and chymotrypsin-like activities was observed (Xu et al., 2004). Further research has demonstrated that both SaPIN2a and SaPIN2b are expressed in floral tissues destined to undergo developmental PCD, suggesting possible endogenous roles in inhibiting trypsin- and chymotrypsin-like activities during flower development (Sin and Chye, 2004).

Transgenic Plants Expressing PIs Against Insect, Pests, and Pathogens

PIs are ubiquitous in plants, esp. in Leguminosae, Gramineae, and Compositae. They are natural, defense-related proteins often present in seeds and induced in certain plant tissues by herbivory or wounding (Koiwa et al., 1997). Transformation of plant genomes with PI-encoding cDNA clones appears attractive, not only for the control of plant pests and pathogens, but also because it can produce PIs useful in alternative systems and because plants can become factories for the production of heterologous proteins (Sardana et al., 1998). That transgenic plants expressing heterogeneous PIs show enhanced resistance to pests is perhaps due to the circumstance that the intrinsic plant PIs are usually not active towards the gut proteases from their own insect pests, a result of the co-evolution of host plants and pests (Jongsma and Bolter, 1997). Also, the intrinsic plant PIs appear present in insufficient amounts to inhibit pest proteinases (Irie et al., 1996).

Table 3 shows some published reports on the production of insect-resistant transgenic plants expressing PIs. The majority of PIs used so far for generating insect-resistant transgenic plants are plant-derived serine PIs like trypsin inhibitors and PIN2s, targeting mainly Lepidopteran pests (Table 3) (Haq et al., 2004; Lawrence and Koundal, 2002). PIs from animal sources expressed in transgenic plants could also help plants enhance their pest-resistance (Brzin and Kidric, 1995) as illustrated with the use of insect PIs (Thomas et al., 1994; 1995a; 1995b).

The use of plant PIs to combat pests and insects, however still has some limitations. In contrast to the successful examples of PIs conferring enhanced pest-resistance in plants, some negative cases have also been reported (Table 4), subsequently leading to decreased interest in the use of PIs to generate insect-resistant transgenic plants (Reeck et al., 1997). The failure resulted from either the ineffectiveness of some PIs on particular insects (Altpeter et al., 1999) or the adaptation of insects to them (Cloutier et al., 2000).

Some reports demonstrated that insects may overcome the resistance of transgenic plants expressing PIs by inducing new proteolytic enzymes that are insensitive to the PI's encoding by a transgene (Jongsma et al., 1995). For example, Colorado potato beetles fed on potato leaves containing high levels of methyl jasmonate-induced PIs were able to produce new proteolytic activities resistant to inhibition by potato PIs (Bolter and Jongsma, 1995). In addition, some insects possess proteases that can digest PI (Girard et al., 1998b).

With the development of transgenic plants expressing PI genes, some doubts and concerns have attracted attention. One of the most important of these is the toxicity to nontarget species, including beneficial insects. Most PIs are safe for mammals because of differences in the organization of the insect and mammalian digestive systems. No acute toxicity was shown in mammalian feeding trials with purified cowpea trypsin inhibitor at a level of 10% of the total protein (Pusztai et al., 1992). The results of in vitro activity assay and feeding trials with artificial diets showed some toxicities of Bowman-Birk soybean trypsin inhibitor (Belzunces et al., 1994) and soybean trypsin inhibitor (Malone et al., 1995) to honey bees. Until now, no report has surfaced on the possible effects of transgenic plants expressing PIs on nontarget species. For those PIs toxic to beneficial insects, the use of a tissue-specific promoter that is inactive in the tissue on which these insects feed could be implemented (Burgess and Gatehouse, 1997).

Table 3. Insect-resistant transgenic plants expressing proteinase inhibitors.

Source gene	Transformed plant	Target insect	Reference
Cowpea trypsin inhibitor (CpTI)	Tobacco	*Heliothis virescens*	Hilder et al., 1987
	Tobacco	*Heliothis virescens*	Boulter et al., 1990
	Tobacco	*Heliothis virescens*	Gatehouse et al., 1993
	Tobacco	*Spodoptera litura*	Sane et al., 1997
	Strawberry	*Otiorhynchus sulcatus*	Graham et al., 1995
	Strawberry	Vine weevil: *Otiorynchus sulcatus* F.	Graham et al., 1997
	Rice	*Sesamia inferens; Chilo suppressalis*	Xu et al., 1996
	Potato	*Lacanobia oleracea*	Gatehouse et al., 1997
	Cotton	*Helicoverpa armigera*	Li et al., 1998
	Cabbage	*Pieris rapae*	Fang et al., 1997
	Pigeonpea	*Helicoverpa armigera*	Lawrence and Koundal, 2001
CpTi and snowdrop lectin	Sweet potato	*Cyclas formicarius*	Newell et al., 1995
	'Jewel' sweet potato	West Indian sweet potato Weevil: *Euscepes postfaciatus*	Golmirizaie et al., 1997
Potato and tomato PIN2	Tobacco	*Manduca sexta*	Johnson et al., 1989
Potato PIN2	Tobacco	*Chrysodeixis eriosoma*	McManus et al., 1994b
	Rice	*Sesamia inferens*	Duan et al., 1996
	Poplar	*Plagiodera versicolora*	Klopfenstein et al., 1997
	Sugarcane	Sugarcane grubs: *Antitrogus consanguineus*	Nutt et al., 1999
Sweet potato (*Ipomea batatas*) trypsin inhibitor	Tobacco	*Spodoptera litura*	Yeh et al., 1997
Tomato proteinase inhibitors I and II	Tobacco	*Manduca sexta* larvae	Johnson et al., 1989
Tomato proteinase inhibitor I	Nightshade	-	Narvaez-Vasquez et al., 1992
	Tobacco	-	
	Alfalfa	-	
Trypsin inhibitor from *Vigna unguiculata*	Tobacco	*Heliothis* *Spodoptera* Diabrotica Anthonomnous Locusts	Hilder et al., 1987
Trypsin inhibitor from barley (CMe)	Indica and japonica rice	Rice weevil: *Sitophilus oryzae*	Alfonso-Rubi et al., 2003
	Barley	*Agrotis ipsilon*	Carbonero et al., 1993
	Tobacco	Lepidoptera	Carbonero et al., 1993
	Wheat	*Spodoptera lituralis*	Alpteter et al., 1999
Rice cysteine PI	Poplar	*Chrysomela tremulae*	Leple et al., 1995
Insect elastase inhibitor	Alfalfa	Thrip (*Frankliniella* spp.)	Thomas et al., 1994
Insect trypsin, chymotrypsin and elastase inhibitors	Cotton tobacco	*Bemisia tabaci*	Thomas et al., 1995a; Thomas et al., 1995b
Ornamental tobacco PIN2	Tobacco	*Helicoverpa punctigera*; *Teleogryllus commodus*	Heath et al., 1997
Sweet potato trypsin inhibitor	Tobacco	*Spodoptera litura*	Yeh et al., 1997
	Cauliflower	*Spodoptera litura*; *Plutella xylostella*	Ding et al., 1998
Mustard trypsin inhibitor 2	Tobacco and *Arabidopsis*	*Spodoptera littoralis*	De Leo et al., 1998
	-	*Spodoptera littoralis* larvae	De Leo and Gallerani, 2002
Mustard trypsin inhibitor (MTI-2)	Tobacco	*Plutella xylostella* (L.)	De Leo et al., 2001
	Arabidopsis	*Mamestra brassicae* (L.)	De Leo et al., 2001
	Oilseed rape	*Spodoptera littoralis* (Boisduval)	De Leo et al., 2001
Nicotiana alata protease inhibitor	Tobacco	*Helicoverpa punctigera*	Heath et al., 1997
	Pea	*Plutella xylostella*	Charity et al., 1999
Barley trypsin inhibitor	Wheat	*Sitotroga cerealella*	Altpeter et al., 1999
Corn cystatin	Rice	Maize grain weevil: *Sitophilus zeamais*	Irie et al., 1996

Table 3. (Continued)

Source gene	Transformed plant	Target insect	Reference
Ornamental tobacco PIN2	Tobacco and pea	*Helicoverpa armigera*	Charity et al., 1999
Oryzacystatin I	Poplar	*Chrysomela tremulae* (Coleoptera: Chrysomelidae)	Leplé et al., 1995
	Potato	Peach potato aphid: *Myzus persicae*	Gatehouse et al., 1996
	Potato	Colorado potato beetle larvae *Leptinotarsa decemlineata*	Lecardonnel et al., 1999
	Oilseed rape	Cabbage seed weevil (Coleoptera: Curculionidae)	Jouanin et al., 1998
	Oilseed rape	*Myzus persicae*	Rahbé et al., 2003
Soybean serine-proteinase inhibitor (C-II)	Potato/tobacco	Coleoptera/Lepidoptera	Schulke and Murdoch, 1983
Soybean (Kunitz) trypsin inhibitor	Tobacco	*Spodoptera litura* larvae	McManus and Burgess, 1999
	Rice	*Nilaparvata lugens*	Lee et al., 1999
Soybean Kunitz trypsin inhibitor (SKTI-4)	Sweet potato	*Cyclas* spp.	Cipriani et al., 1999
Soybean Kunitz, C-II and PI-IV inhibitor	Potato/tobacco	*Spodoptera littoralis*	Marchetti et al., 2000
American black nightshade proteinase inhibitor SaPIN2a	Lettuce	-	Xu et al., 2004
Arabidopsis thaliana cysteine proteinase inhibitor	White poplar (*Populus alba* L.)	Chrysomelid beetle: *Chrysomela populi*	Delledonne et al., 2001

Table 4. Transgenic plants expressing proteinase inhibitors lacking insect resistance.

Source gene	Transformed plant	Target insect	Reference
Tomato proteinase inhibitor I	Tobacco	*Manduca sexta*	Johnson et al., 1989
Potato PIN2	Tobacco	*Spodoptera litura*; *Thysanoplusia orichalcea*	McManus et al., 1994b
	Tobacco	*Spodoptera exigua*	Jongsma et al., 1995
Giant taro PI	Tobacco	*Helicoverpa armigera*	Wu et al., 1997
Rice Oryzacystatin I	Oilseed rape	*Ceutorhynchus assimilis*	Girard et al., 1998a
	Potato	*Leptinotarsa decemlineata*	Cloutier et al., 2000
Barley trypsin inhibitor	Wheat	*Melanoplus sanguinipes*	Altpeter et al., 1999

No transgenic plant expressing PIs has yet been commercialized (Gatehouse et al., 1997). The first insect-resistant transgenic corn, cotton and potato expressing *Bacillus thuringiensis* (*Bt*) toxins were released commercially in the USA in 1995 (Jouanin et al., 1998; Schuler et al., 1998). Transgenic cotton expressing *Bt* toxin has been cultivated on a large scale in northern China every year. This may be due to a less consistent and less pronounced pest resistance exhibited by plants expressing PI compared to those produced by expressing *Bt* toxin under field conditions (Hoffmann et al., 1992). At present, the main strategy to develop insect-resistant plants via genetic engineering is based on the use of *Bt* toxin genes. Current transgenic *Bt* crops expressing the Cry protein genes (crystal proteins or endotoxins) target key pests and also those resistant to conventional pesticides. Now *Bt* makes up to 98% of all biopesticides and represents the quasiexclusive source of pest-resistance genes for the development of transgenic plants. However, there are limitations on the use of transgenic *Bt* plants as well. Increased persistence of the *Bt* toxin within the plant throughout the growing season selects intensely for insect resistance (Moar et al., 1995; Haq et al., 2004). Also, the range of insects which can be controlled by *Bt* toxins is relatively narrow (Haq et al., 2004).

Lettuce (Lactuca sativa L.) and its Transformation

Lettuce, *Lactuca sativa* L., is a cool-season, leafy vegetable that belongs to the largest dicotyledonous family in the plant kingdom, the *Asteraceae* (*Compositae*). Lettuce is in the subfamily *Cichorioideae*, the tribe *Lactuceae*, the genus *Lactuca* (Ryder, 1999).

Lettuce, an economically important vegetable crop, is grown globally. Lettuce is a fairly hardy, cool-weather vegetable that thrives when the average daily temperature is between 60 and 70°F. It should be planted in early spring or late summer. At high temperatures, growth is stunted, and the leaves may be bitter, and the seed stalk forms and

elongates rapidly. Some types and varieties of lettuce withstand heat better than others (USDA, 1998; Ryder, 1999). The nutritional value of lettuce varies with the variety. Lettuce in general provides small amounts of dietary fiber, some carbohydrates, a little protein, and a trace of fat. Its most important nutrients are vitamin A and potassium. The vitamin A comes from beta carotene, whose yellow-orange is hidden by green chlorophyll pigments. Beta carotene, of course, is converted to vitamin A in the human body. The darker green, the more beta carotene (USDA, 1998; Ryder, 1999). According to the American Cancer Institute and the American Cancer Society, foods rich in vitamin A and C (antioxidants) offer protection against some forms of cancer. Along with other phytochemicals, antioxidants reduce the risk of cancer of the respiratory system and intestinal tract. Lettuce, except iceberg, is also a moderately good source of vitamin C, calcium, iron, and copper. The spine and ribs provide dietary fiber, while vitamins and minerals are concentrated in the delicate leaf portion (USDA, 1998; Ryder, 1999).

The greatest producer and consumer of lettuce is the USA (113, 000 hectares, total value $ 1.6 billion in 1997) (USDA, 1998). Large areas of lettuce are also grown in Western Europe, Australia, Japan, Israel, South and Central America, Africa and China (Ryder, 1999). One of the main problems in commercial production of lettuce is insect pests, particularly the cabbage looper, *Trichoplusiani* (Hubner). In the USA, as many as fifteen insecticide applications per season are required to control this very destructive pest in Arizona and California (Pink and Keane, 1993; Barbour, 1999). Insect-resistant transgenic plants offer an alternative strategy of pest control that may eliminate the reliance on chemical pesticides (Estruch, 1997) and have proven to be a promising approach.

Since the first report on the *Agrobacterium tumefaciens*-mediated transformation of lettuce (Michelmore et al., 1987), more than ten reports of the production of transgenic lettuce have been published (Table 5). Most of these studies used the *Agrobacterium*-mediated transformation method with cotyledons as explants. While a lot of work on transgenic lettuce involves transferring reporter or marker genes (Michelmore et al., 1987; Chupeau et al., 1989; Enomoto et al., 1990; Torres et al., 1993; Curtis et al., 1994; McCabe et al., 1999), several agronomically important traits have also been transferred to lettuce (Curtis et al., 1996; Dinant et al., 1997; Curtis et al., 1999). Xu et al. (2004) developed transgenic lettuce expressing heterogeneous proteinase inhibitor SaPIN2a. The finding that heterogeneously expressed SaPIN2a in transgenic lettuce inhibits plant endogenous protease activity further indicates that SaPIN2a regulates proteolysis and could be potentially exploited for the protection of foreign protein production in transgenic plants (Xu et al., 2004). So far, however, no insect-resistant transgenic lettuce has been developed although some unpublished studies on transgenic lettuce expressing PIs have been mentioned in review articles (Burgess and Gatehouse, 1997).

Chinese Flowering Cabbage (*Brassica campestris ssp. parachinensis*) *and its Transformation*

Chinese flowering cabbage (*Brassica campestris* ssp. *parachinensis*) is in the genus *Brassica* of the family *Cruciferae.*

Brassica species have high productivity, good yield, and good agronomic characteristics. Many of these species are used for food, as oils, and as animal feed, so breeding programs have involved innovative techniques to assist the release of new cultivars. Chinese flowering cabbage is a native of Guangdong Province and is grown and consumed virtually all year-round throughout Southeast Asia. It has other common names, such as tsoi sum and choy sum. It is perhaps the most common form of Chinese greens sold in the green grocery, and is distinguished by the small yellow flowering heads that protrude from the bunch of leaves. The leaves are yellowish-green and distinguishable from Chinese Broccoli, which has white flowers and bluish-green leaves.

Because of its pleasant taste and cooking qualities, Chinese flowering cabbage has become the most common leafy vegetable, also a staple local vegetable and is widely cultivated in Southeast Asia, especially in South China, including Hong Kong (Opena et al., 1988; Wong et al., 1996). Like lettuce and many other vegetables, Chinese flowering cabbage also suffers from many diseases and insect pests. The major diseases of concern include clubroot (*Plasmodiophora brassicae*), downy mildew (*Peronospora parasitica*), white rust or white blister (*Albugo candida*), and edema (oedema). The major pests include aphids (*Brevicoryne brassicae*), the large and small cabbage white butterflies (*Pieris rapae*), the diamond back moth (*Plutella xylostella*), and the green looper (*Chrysodeixis eriosoma*), and snails and slugs (Class Gastropoda) (Xiang et al., 2000).

Chinese flowering cabbage, like other subspecies of *Brassica campestris*, is known to be recalcitrant to in vitro shoot regeneration (Murata and Orton, 1987; Jain et al., 1988; Narashimhulu and Chopra, 1988). Its regeneration procedure has been established with cotyledon and hypocotyl explants by using ethylene inhibitor $AgNO_3$ in the medium (Chi et al., 1990). Microspore culture is used as an alternative to conventional breeding and is used in many *Brassica* breeding programs, including Chinese flowering cabbage (Wong et al., 1996). In contrast to lettuce, only one report of successful transformation of Chinese flowering cabbage has been published (Xiang et al., 2000).

American Black Nightshade (Solanum americanum Mill.)

American black nightshade (*S. americanum* Mill., Synonyms/other Latin names: *S. caribaeum* Dun. (see), *S. linnaeanum* Hepper & Jaeger (see), *S. nodiflorum* Jacq. (see), *S. nigrum* var. *americanum* L. (Mill.) Schulz (see), *S. nodiflorum* ssp. *nutans* Jacq. R.J.F. Hend.; Common name(s): glossy nightshade, American black nightshade, black nightshade, garden nightshade, nightshade, yerba mora negra, Maria preta) is a member of *Solanum* sect. in the genus *Solanum* of the family Solanaceae.

Solanum (Solanaceae), a group of annual or short-lived perennial herbaceous weeds, is found on roadsides, farmlands, and on the edges of villages and towns throughout temperate and tropical zones of the world (Ma, 1995). Some members of *Solanum* sect. *Solanum*, commonly called "black nightshade," are serious weeds (Callihan et al., 1990) while many of them are used widely as leafy herbs, and somewhat less commonly as a source of fruits or for medicinal /culinary purposes (Schilling et al., 1992). *Solanum americanum*, a diploid species widespread in tropical and subtropical areas through the world, is often confused with *S. nigrum* (black nightshade, common nightshade, garden nightshade, stubble-berry) (Schilling et al., 1992; Ma, 1995), a hexaploid species which has been documented in Hong Kong (Thrower, 1984).

Solanum americanum has umbelliform inflorescences, shiny fruits, smaller anthers (1-2 mm long), pollen (19-27 μm in diameter) and seeds (1.2-1.6 mm long), and often has prominently reflexed sepals in fruits (Schilling et al., 1992); *S. nigrum* has racemiform inflorescences, dull fruits, larger anthers (1.8-2.5 mm long), pollen (25-35 μm in diameter) and seeds (1.8-2.2 mm long), and sepals that generally adhere to the fruit.

Solanum americanum, originated from the Americas, is extremely tolerant to aridity. From an evolutionary viewpoint, this weed would have evolved to resist insects endemic to this region (De Leo et al., 2002). *Solanum americanum* is a rich source of proteinase inhibitors (Brzin and Kidric, 1995), and also a suitable source for cloning PIN2 cDNAs. In fact, two cDNA clones encoding PIN2, designated *SaPIN2a* and *SaPIN2b*, have already been isolated by screening *S. americanum* cDNA library prepared from wounded leaves using a tomato *PIN2* cDNA (Graham et al., 1985a, 1985b; Xu et al., 2001) as a heterologous hybridization probe.

Since this weed has evolved separately from the insect pests of vegetables, the PIs from this non-host plant could effectively inhibit the gut proteinases of vegetable pests (Harsulkar et al., 1999) and could potentially be used in generating insect-resistant transgenic vegetables. It would also take a much longer time for insects to develop tolerance to the PIN2 from this novel source that insects have not previously encountered.

Conclusions and Future Perspectives

The majority of proteinase inhibitors studied in the plant kingdom originate from three main families, namely Leguminosae, Solanaceae, and Gramineae. Plant PIs are well known to play a potent defensive role against predators and pathogens. Diverse endogenous functions for these proteins have already been proposed, ranging from regulators of endogenous proteinases to storage proteins, but evidence for many of these roles is partial or confined to isolated examples (Mosolov et al., 2001; Lawrence and Koundal, 2002; Birk, 2003; Shewry, 2003). In addition, many plant PIs have been shown to act as defensive compounds against insects by direct assay or by expression in transgenic crop plants, and a body of evidence for their role in plant defense has accumulated consistently (Lawrence and Koundal, 2002). The role and mechanism of action for most of these inhibitors have been, or are being, studied in detail, and their respective genes have been isolated. These genes have been used for the construction of transgenic crop plants to be incorporated in integrated pest management programs (Lawrence and Koundal, 2002; Haq et al., 2004). Given the number of pesticidal proteins involved in host plant defense, effective pest control by this strategy will presumably result from the co-expression of numerous determinants, each of which

Table 5. Summary of work reported on the transformation of lettuce.

Lettuce cultivar	Transgene*	Explant	Transformation method	Reference
Cobham Green	NPT II	Cotyledon	*Agrobacterium*	Michelmore et al., 1987
Ardente	CAT, NPT II	Mesophyll protoplasts	Electroporation	Chupeau et al., 1989
Kayser	GUS, NPT II	Cotyledon	*Agrobacterium*	Enomoto et al., 1990
South Bay	GUS, NPT II	Cotyledon	*Agrobacterium*	Torres et al., 1993
Lake Nyah, Mantillia, etc. (13 cultivars)	GUS, NPT II	Cotyledon	*Agrobacterium*	Curtis et al., 1994
Lake Nyah	Glucanase	Cotyledon	*Agrobacterium*	Curtis et al., 1996
Asgrow proprietary breeding line D and line M	Nucleocapsid protein of TSWV	Cotyledon	*Agrobacterium*	Pang et al., 1996
Girelle, Jessy, Cocarde	LMV CP	Cotyledon	*Agrobacterium*	Dinant et al., 1997
Diana	Maize Ac transposase and Ds	Cotyledon and leaf	*Agrobacterium*	Okubara et al., 1997
Flora, Cortina, Luxor, Evola	Nitrate reductase	Cotyledon	*Agrobacterium*	Curtis et al., 1999
Great Lake #118	ipt, hpt, luc	Cotyledon	*Agrobacterium*	Kunkel et al., 1999
Raisa	GUS, NPT II	Cotyledon	*Agrobacterium*	McCabe et al., 1999
Great Lakes No. 118	*SaPIN2a*	Cotyledon	*Agrobacterium*	Xu et al., 2004

*CAT: chloramphenicol acetyl transferase; GUS: β-glucuronidase; hpt: hygromycin phosphotransferase; ipt: isopentenyltransferase; LMV CP: lettuce mosaic potyvirus coat protein; luc: firefly luciferase; NPT II: neomycin phosphotransferase II; TSWV: tomato spotted wilt virus.

could be custom engineered by directed molecular evolution to maximize its effectiveness against specific pests.

The genetic background of a variety, the weather, soil fertility, moisture stress, or insect injury can all influence the effectiveness of plant-produced pesticides or of any other gene product of the crop plant (Benedict, 2003). It seems that plant genetic engineering has to be adopted for maximum benefits with a minimum input to meet the increasing demand for food from the burgeoning human population worldwide, considering the decreasing amount of cultivable land. With the development of disease-, insect-, and drought-resistant crops, genetic engineering has addressed at least some of the environmental problems associated with conventional agriculture. Though the use of transgenic plants may also have some effects on environmental and public health, antibiotic resistance and gastrointestinal problems have been addressed to some extent by the use of herbicide-tolerant genes and studies on the nutritional effects of proteinase inhibition on mammals (Haq et al., 2004).

Additionally, other factors may affect transgenic expression. Because gene expression may be developmentally regulated, some genes are differentially expressed. Gene silencing may occur even if the gene can be integrated into the target site of the host. Multiple gene copies, not only a single gene copy, may be incorporated, and the gene may be incorporated at different sites. In addition, environmental conditions such as heat, water, and stress, effect transgenic expression (Haq et al., 2004).

The insect midgut reportedly contains an estimated 1020 different proteases (Bown et al., 1997). These are differentially regulated and cannot all be inhibited by plant's PIs (Broadway, 1996). Therefore, to achieve an effective pest control strategy it is very important to orchestrate the expression of different inhibitors in a concerted manner. With the development of transgenic, insect- and pest-resistant crop varieties, the proteinase inhibitor genes will make a promising contribution towards maximizing yields and minimizing losses due to insects and pests. We can anticipate a number of promising possibilities for pest control through insecticidal genes. All need to be explored and prudently tapped for their implementation in integrated pest management programs.

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