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Plant proteinase inhibitors: A defensive response against insects. (Review).

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Plants protect themselves against pests using their wide chemical defense arsenal. Among several defense proteins, proteinase inhibitors appear to be an important group. Proteinase inhibitors are widely present in plants and they are often found in storage organs. They are known to be inducible in plants by injuries, such as insect damage. Because these proteins inhibit digestive enzymes of insect larvae and microbial proteases, they may be considered as mechanisms to improve the plant defense against pests. In recent years, growing research on plant proteinase inhibitors has confirmed their important role in plant defense, although several aspects are still controversial. Although many plants have related proteinase inhibitors, which have been shown to affect metabolism and/or development of different insects, these plants do not seem to share a common inhibitor induction mechanism. This is an emerging field and much work is yet to be done.

***Key words**: insect pest control, protease inhibitor, proteinase inhibitor, plant defensive genes.*

INTRODUCTION

Proteins that inhibit proteolytic enzymes are widespread in nature. They are known to interact highly specifically with proteinases in a competitive way. These proteinase inhibitors (PIs) are often found in many organs and tissues of plants, animals, and microorganisms. They have been studied for several reasons: as animal digestive enzymes inhibitors found in agricultural crops, as a tool to understand the mechanism of protein-protein interactions, and in the medical field, as possible therapeutic agents. In the last decades, there have been new interesting study areas of PIs: their possible roles in plant metabolism and as contributors to plant defense against insects and pathogens.

General knowledge about PIs has been reviewed elsewhere (Bode and Huber, 1992; Laskowski and Kato, 1980; Richardson, 1977; Ryan, 1973, 1981, 1990). In the last years, literature has focused on the characterization of PIs from plants and their gene expression. This review aims at summarizing some aspects of plant PIs, specially recent hypotheses on their role in plant protection.

GENERAL PROPERTIES

Proteinase inhibitors found in plants are typically polypeptides and proteins. In contrast with those found in animals (Laskowski and Kato, 1980), PIs from plants

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are not present as glycoproteins, except the reported case of a papain inhibitor (Ryan, 1981). Sizes of plants PIs vary from 4,000 to 80,000 kDa, but most of them fall in the range of 8,000 to 20,000 kDa.

Proteinase inhibitors have a reactive site which binds to the catalytic-site residues of the cognate proteinase in a similar manner to that of substrates. Most PIs have their reactive sites as an exposed 'binding loop', which is stabilized by interactions between residues flanking the reactive site through many hydrogen bonds and generally by disulfide bonds in the hydrophobic inhibitor core (Bode and Huber, 1992). In some inhibitors (*e.g.*, potato inhibitor I) the binding loop is stabilized by electrostatic hydrogen bonds, such as in two parallel chains of Arg (Bode and Huber, 1992). Plant PIs are quite stable molecules and are often resistant to heat, pH extremes and to proteolysis by proteases, even by those proteases they do not inhibit (Ryan, 1981). Their stability has been attributed in part to the high proportion of half-cysteine residues present as disulfide cross-links, and to non covalent interactions (Laskowski and Kato, 1980; Ryan, 1981). It is usual to find gene duplication resulting in inhibitors having two nearly identical halves, each with an active site, a so-called "double-headed" inhibitor capable of inhibiting two molecules of enzyme at the same time.

OCCURRENCE, DISTRIBUTION AND PHYSIOLOGICAL ROLES

Although plant PIs are distributed throughout the plant kingdom, they are specially abundant in those tissues which form important sources of food, such as the seeds of the Leguminosae, potato tubers and most cereal grains.

The cellular location of many of the plant PIs is still uncertain. Some of these are known to form part of the vacuolar protein bodies (*e.g.*, inhibitor I in Solanaceae) (Walker-Simmons and Ryan, 1977). Trypsin inhibitors are associated with protein bodies of leguminous seeds, but the possibility that some inhibitors could have cytoplasmic location has not been ruled out (Richardson, 1977). PIs have been found in the cotyledons of soybean (Ryan, 1973), and cereal endosperms and embryos (Boisen and Djurtaft, 1982).

It has been suggested that PIs may have an important role in the regulation of endogenous proteinases (Ryan, 1973). Although PIs from some seeds have not shown inhibition of endogenous proteinases, there is some evidence indicating that lettuce and barley seeds do inhibit those proteinases (Kirsi and Mikola, 1971; Ryan, 1973). Barley seeds contain three types of PIs: one that inhibits trypsin, another that inhibits chymotrypsin and microbial proteinases, and other that inhibits endogenous proteinases (Kirsi, 1973; Boisen *et al*, 1981). During germination, the latter decreases as proteolysis begins to increase (Kirsi and Mikola, 1971; Kirsi, 1974).

Since PIs are present in large amounts in tubers and seeds, they may be considered storage proteins. In barley grains and potato tubers, PIs represent up to 10% of total proteins (Kirsi and Mikola, 1971; Ryan, 1973). This storage role may also be valid in tissues other than storage organs, such as leaves, shoots, roots, flowers and sprouts. Kirsi (1974) and Kirsi and Mikola (1977) showed that, after germination, there is a basal activity of some iso-inhibitors that remains in barley leaves and roots, while others disappear within the early stages of growth.

A more complex function is against the attacks of insects and microorganisms. It seems that those PIs found as storage proteins in aerial tissues are also involved in plant protection. This role will be discussed later, as it appears to be an interesting emerging field.

PIs PRESENT IN PLANTS

Endopeptidases or proteinases cleave internal peptide bonds and are classified into four classes depending on the nature of their active site: serine proteinases (trypsin, chymotrypsin, thrombin, plasmin, elastase), cysteine or sulphydryl proteinases (papain, bromelain, ficin, cystatins), aspartic acid proteinases (pepsin, renin, cathepsin E) and

TABLE I

Plant proteinase inhibitor families

	Inhibitor	Protease inhibited	Plant	Reference
1	Soybean trypsin inhibitor (Kunitz) family			
	SBTI	Trypsin	Soybean (*Glicine max*)	Richardson, 1977; Gotor *et al*, 1995
2	Bowman-Birk inhibitor family			
	SBI B	Trypsin / chymotrypsin	Soybean	Odani *et al*, 1977
	SBI C-II	Trypsin / chymotrypsin / elastase	Soybean	Odani *et al*, 1977
	SBI D-II / E-I	Trypsin	Soybean	Odani *et al*, 1977
	LBI-I / IV	Trypsin / chymotrypsin	Limabean (*Phaseolus lanatus*)	Richardson, 1977
	GBI-II	Trypsin / subtilisin / elastase	Garden bean (*P. vulgaris*)	Richardson, 1977
	ABI-I	Trypsin / chymotrypsin	Adzuki bean (*P. angularis*)	Bode and Huber,1992
	PI-A-II	Trypsin / chymotrypsin	Peanut (*Arachis hypogea*)	Bode and Huber, 1992
	CpI	Trypsin / chymotrypsin	Chickpea (*Cicer arietinum*)	Richardson, 1977
	CpTI	Trypsin / chymotrypsin / subtilisin	Cowpea (*Vigna unguiculata*)	Vartak *et al*,1980
	MBTI	Trypsin / chymotrypsin / subtilisin	Mung bean (*Vigna radiata*)	Bode and Huber, 1992
	BBTI	Trypsin / chymotrypsin / subtilisin	Broad bean (*Vicia faba*)	Ryan, 1990
	ATI	Trypsin	Alfalfa (*Medicago sativa*)	Brown and Ryan, 1984
	PTI-I / II	Trypsin	Pea (*Pisum sativum*)	Domoney *et al*, 1993
	BBI-M	Trypsin	Maize (*Zea mais*)	Eckelkamp *et al*, 1993
	WTI-I / II	Trypsin	Wheat (*Triticum* spp.)	Odani *et al*, 1986
	BEmTI I / II	Trypsin / chymotrypsin	Barley (*Hordeum vulgare*)	Boisen and Djurtoft, 1982
3	Potato inhibitor I family			
	PI-I	Chymotrypsin / subtilisin	Potato (*Solanum tuberosum*)	Gurusiddaiah and Ryan, 1972
			Tobacco (*Nicotiana tabaccum*)	Gurusiddaiah and Ryan, 1972
	PI-I	Chymotrypsin	Tomato (*Licopersicum esculentum*)	Green and Ryan, 1972
	ATSI	Trypsin / chymotrypsin / subtilisin / cathepsin G	Amaranth (*Amaranthus caudatus*)	Hejgaard *et al*, 1994
	CI-I / II	Chymotrypsin / subtilisin	Barley / wheat / maize / sorghum (*Sorghum* spp.)/ triticale / rye (*Secale cereale*)	Mosolov and Shul'gin, 1986; Hejgaard, 1981
	MPI	Serine proteases	Maize	Cordero *et al*, 1994
4	Potato inhibitor II family			
	PI-II a / b	Trypsin / chymotrypsin	Potato / tomato	Gustafson and Ryan, 1976
	TTI-I	Trypsin / chymotrypsin	Tobacco	MacManus *et al*, 1994
5	Squash inhibitor family			
	CMTI-I / II	Trypsin	Squash (*Cucurbita maxima*) / summer squash (*C. pepo*) / cucumber (*Cucumis sativum*)	Kupryszewski *et al*, 1994
6	Barley trypsin inhibitor family			
	BTI	Trypsin	Barley	Odani *et al*, 1983
		Trypsin	Sorghum / maize / oat (*Avena sativa*)	Boisen, 1983
		Trypsin / subtilisin	Barley / wheat / rye	Mosolov and Shul'gin, 1986; Boisen, 1983.
7	Bifunctional inhibitors			
	Ragi I-2	α-Amylase / trypsin	Ragi (*Eleusin coracan*)	Ryan, 1990
		α-Amylase	Barley / wheat / maize	Boisen, 1983; García-Olmedo *et al*, 1987

TABLE I (Continuation)

Plant proteinase inhibitor families

	Inhibitor	Protease inhibited	Plant	Reference
8	Sulphydryl inhibitor / cystatins family			
	PCPI	Papain/ chymopapain / ficin	Potato	Rodis and Hoff, 1984
	Cys-PIs	Papain	Pineapple (*Ananas sativus*)/ maize / cowpea / tomato / tobacco / mung bean / wheat / barley / rye	Rele *et al*, 1980; Ryan, 1990
		Bromelain	Pineapple	Laskowski and Kato, 1980
		Papain / ficin / bromelain	*Bauhinia*	Laskowski and Kato, 1980
		Papain	Canola (*Brassica napus*)	Bodnaryk and Rymerson, 1994
	OC-I / II	Papain / cathepsin H	Rice (*Oryza sativa*)	Michaud *et al*, 1993
	CC-I	Papain	Maize	Abe *et al*, 1992
9	Metallocarboxypeptidase inhibitor family			
	CPI	Carboxypeptidase A,B	Potato / tomato	Ryan, 1973
10	Potato carboxypeptidase inhibitor family			
	Acid PI	Cathepsin D / trypsin / chymotrypsin	Potato	Laskowski and Kato, 1980
		Pepsin	*Bauhinia*	Laskowski and Kato, 1980

metallo-proteinases (carboxypeptidases A and B, aminopeptidases, cathepsin D) (Richardson, 1977; Ryan, 1990). Plants have inhibitors for serine and acid and sulphydryl proteinases. Although aminopeptidases inhibitors have not yet been reported from plants or animals, they have been found in microorganisms (Richardson, 1977). Plant PIs inhibit proteinases of animal, bacterial and fungal origins, and occasionally inhibit plant proteinases (Ryan, 1973).

The class to which a specific PI belongs is determined by its ability to inhibit specific proteinases and by its similarity to well characterized inhibitors. In plants, at least ten different inhibitor families have been proposed (see Table I). They are well differentiated from those families of animal protease inhibitors, although some share similar mechanisms of action. Classifying all known plant PIs has been difficult. The most representative PIs of each family were described first. Then, new PIs appeared and were found to share homologies with already known PIs. This has happened often with the cereal proteinase inhibitors, since they inhibit more than one inhibitor type and share structural similarities with other families, such as the Potato inhibitor I and Bowman-Birk inhibitors. Other examples are the bifunctional protease/α-amylase inhibitors present in cereal grains, some of which may be considered within the Kunitz family, and others within the Barley trypsin inhibitor family. Comprehensive studies of cereal protease/α-amylase inhibitors have been published (García-Olmedo *et al*, 1987, 1992; Carbonero *et al*, 1994). In Table I we have attempted to classify plant PIs according to the closest relation among PIs. Although this division has been proposed during the last two decades (see Ryan 1973, 1990; Laskowski and Kato, 1980; Garcia-Olmedo *et al*, 1987), it does not pretend to be unique and may vary by new findings. For each inhibitor family, we did not include all the inhibitors known to date, citing only the best known examples.

The comparison of the amino acid sequences of PIs has provided not only information on structural and functional features, but has also revealed aspects of the evolution of plant PIs. People who have studied the Bowman-Birk family agree that double-headed inhibitors from legumes come from a common ancestral gene (Odani and Ikenaka, 1977; Laskowski and Kato, 1980). The same may have happened with other inhibitor families. Another feature of homology among PIs resides in the reactive

site (P_1 - P_1' residues). For better known trypsin inhibitors, the first residue (P_1) is Arg or Lys, and for chymotrypsin inhibitors is Leu, Phe, Trp or Tyr. A substitution of one of this amino acids may be carried out in the laboratory. Then, an inhibitor against trypsin may be converted into another against chymotrypsin (Laskowski and Kato, 1980). This modification may have happened in nature throughout evolution, resulting in inhibitors with different specificities within the same family.

PIs IN PLANT DEFENSE

Several gene products are involved in plant defense (Bowles, 1990; Staskawicz *et al*, 1995). Among these, PIs have become an important emerging group. Induction of PIs has been described upon attack by herbivorous insects (Green and Ryan, 1972) or by fungal infection (Peng and Black, 1976; Rickauer *et al*, 1992). In many cases, these proteins have also been found to be inducible by wounding, both locally at the site of injury and systemically in the whole plant (Graham *et al*, 1986; Peña-Cortés *et al*, 1988). New reports appeared about PIs induction in maize (Eckelkamp *et al*, 1993; Cordero *et al*, 1994), and tobacco (Linthorst *et al*, 1993; Jongsma *et al*, 1994; MacManus *et al*, 1994).

Systemic induction of PIs led to the proposal of the existence of a "wound signal" which carries the information from the damaged tissues to the rest of the plant, where expression of PI genes also takes place. In this process, chemical elicitors, physical events and some plant hormones have been involved (Ryan, 1992 and references therein). The identity of this signal, also called proteinase inhibitor inducing factor (PIIF), has been subject of discussion. Some candidates were refuted to be good mobile signals (Baydoun and Fry, 1985; Ryan, 1992), while others like the polypeptide systemin have been proved to be the contrary (McGurl *et al*, 1992 and Pearce *et al*, 1991). Systemin is well characterized in the Solanaceae and has been suggested to participate in the proposed signal transduction pathway (Farmer and Ryan, 1992). Recent experiments with transgenic tomato support its role in the resistance against insects (Orozco-Cardenas *et al*, 1993). Transgenic tomato, which constitutively overexpresses the prosystemin gene, produces inhibitors I and II activating its biosynthetic pathway (McGurl *et al*, 1994; Constabel *et al*, 1995). Other studies on the translocation of systemin also help to demonstrate its participation in systemic signalling (Narvaez-Vasquez *et al*, 1994).

According to what was described in some wounding experiments (Graham *et al*, 1986; Peña-Cortes *et al*, 1988), systemic induction of PIs must, therefore, involve rapid travel of the signal. Phloem transport has been suggested (Peña-Cortés *et al*, 1988), but some authors stated that probably electric signalling (Wildon *et al*, 1992) and hydraulic mechanisms (Malone 1992; Boari and Malone, 1993) may be involved. The latter refers to propagating changes in water pressure which are triggered by wounds. It also involves a rapid mass flow from the wound site. Thus, the chemical agent or PIIF could be carried in the xylem-borne mass flow throughout the plant (Malone *et al*, 1994). This hydraulic mechanism has been demonstrated in various plant species (Boari and Malone, 1993). There are also arguments against the alternative mechanism of electrical signalling (Alarcon and Malone, 1994).

Additionally, the participation of abscisic acid (ABA), auxins and jasmonic acid and its methyl ester has also been demonstrated. ABA has been shown to participate in the induction of inhibitor II mRNA in potato and in ABA-deficient mutant tomato (Hildmann *et al*, 1992, Peña-Cortés *et al*, 1989). The levels of mRNA are similar to those found in wounded plants. However, in non mutant plants, ABA induces this inhibitor only in potato. The reason is not understood yet. Even though some experiments suggested induction of an inhibitor II-class gene by auxins (Kernan and Thornburg, 1989; Taylor *et al*, 1993), the participation of auxins is not widely accepted (Thornburg and Li, 1990; Sanchez-Serrano *et al*, 1991). Ethylene participation in PIs induction was also discarded (Sanchez-Serrano *et al*, 1991; Rickauer *et al*, 1992).

Jasmonic acid (JA) and its volatile ester methyl jasmonate strongly induce the accumulation of PIs I and II when applied to potato and tomato leaves (Farmer *et al*, 1992). Moreover, methyl jasmonate can act as a volatile signal inducing PI accumulation in nearby plants (Farmer and Ryan, 1990). Jasmonic acid is known to be a stress modulator (for a review see Parthier, 1990; Staswick, 1992; Sambder and Parthier, 1993; Reinbothe *et al*, 1994) and has shown to induce the so-called jasmonate induced proteins (JIPs) in various plant species (Reinbothe *et al*, 1994). Besides the induction of PIs in solanaceous plants, JA induces the synthesis of some polypeptides involved in the defense against pathogens in barley, such as thionins (Andresen *et al*, 1992) and some ribosome-inactivating proteins (RIPs) (Reinbothe *et al*, 1994). Thionins are small polypeptides which have antifungal activity (Bohlmann and Apel, 1991). RIPs seem to be involved in local pathogen resistance, a mechanism similar to the hypersensitive response. Simultaneously, JA lowers or even shuts down the expression of photosynthetic and other genes (Reinbothe, 1994, and related references therein). In soybean, JA and its methyl ester increase mRNA levels of other wound-responsive genes such as chalcone synthase and proline-rich cell wall protein (Creelman *et al*, 1992).

With all these participant molecules, few models have been proposed to understand this signal transduction pathway. In general, the systemic or localized signals may switch on the synthesis of jasmonic acid in cells, which would be responsible for PIs gene activation. This idea is supported by the ability of several intermediates of JA metabolism (Farmer and Ryan, 1992) and some JA-related molecules to induce this plant defense mechanism (Ishikawa *et al*, 1994).

The defensive role of PIs was first described in potato and tomato. Much of the knowledge concerning the induction of PIs was stated for the Solanaceae. This family is still the best system for the study of PIs. Unfortunately, it seems that regulation and induction of PIs upon wounding or pathogen attack is not an identical feature among plant species (Linthorst *et al*, 1993; Jongsma *et al*, 1994; Peña-Cortés *et al*, 1988). Thus, attempts to have an integrated model for higher plants have been unsuccessful.

Recently, a Bowman-Birk trypsin inhibitor-related protein was found to accumulate by wounding in maize (Eckelkamp *et al*, 1993). The sequence of this protein demonstrates a strong homology with the cereal double-headed (Bowman-Birk-type) inhibitors, specially in the reactive site. The authors demonstrated the translocation of the transcript between organs of the maize seedling, but this systemic response seems to happen mainly in an acropetal direction. A similar result was also obtained from maize, where an inhibitor (MPI) is induced by fungal infection, mechanical wounding, abscisic acid and methyl jasmonate (Cordero *et al*, 1994). This recent report demonstrated both local and systemic induction of the MPI gene expression. This response is similar to the dicot system. The amino acid sequence of this protein reveals homology with amino acid sequences from the PI-I family. The highest homology (60%) is found with the barley inhibitors CI-1 and CI-2.

PROTECTIVE ROLE OF PIs AGAINST INSECTS

Insects have several protein digestive enzymes, but usually one predominates. Serine proteinases are often present as the main digestive enzymes of insect midguts with neutral or alkaline pH, and cysteine and aspartic acid proteinases in more acidic guts (Boulter, 1993). One may think that any protease inhibitor should affect many insects from different families. Among insects, there is variability in the their main proteases. Not all the insects appear to have digestive proteases, depending on sugars and free amino acids absorbed from the phloem sap. In some aphids, however, trypsin, cathepsin and other basic peptidases besides amylases and cell wall degrading pectinases have been found (Auclair, 1963).

Several examples of the effect of PIs on insects have been published, but the scarce information on the impact of the inhibitors on insect growth and development is tied to the lack of detailed knowledge of insect

proteinases. Tomato PI I affects the beet armyworm *Spodoptera exigua* (Broadway *et al*, 1986) and increases natural defenses against *Manduca sexta* larvae in transgenic tobacco (Johnson *et al*, 1989). In this latter system, cowpea trypsin inhibitor (Bowman-Birk) also affects *Heliothis virescens* (Hilder *et al*, 1987) and *Helicoverpa zea* (Hofmann *et al*, 1992). This latter inhibitor has effects on the metabolism and development of the burchid beetle *Callosobruchus maculatus* (Gatehouse and Boulter, 1983). Growth of larvae of *Heliothis zea* and *S. exigua* was inhibited with 10% purified soybean trypsin inhibitor and potato inhibitor II in diets (Broadway and Duffey, 1986). Other studies have demonstrated deterrent activity of serine PIs on growth and development of the cricket *Teleogryllus commodus* (Burgess *et al*, 1994) and the codling moth *Cydia pomonella* (Markwick *et al*, 1995). Coleoptera insect pests use often cysteine proteases for protein digestion (Michaud *et al*, 1993; Wolfson and Murdock, 1987). Thus, cysteine inhibitors were also tested against these pests. A greater cysteine inhibitor content in *Brassica napus* leaves diminishes the feeding rate of the flea beetle *Phyllotetra cruciferae* (Bodnaryk and Rymerson, 1994). Rice cystatins gave good results inhibiting cathepsin H from the Colorado potato beetle *Leptinotarsa decemlineata* (Michaud *et al*, 1993). It is thought that transformation of potato with rice cystatin genes could represent an attractive approach for the control of the beetle. Such a strategy could be useful to the extent that these cystatins do not interfere with proteins involved in tuber proteins breakdown (Michaud *et al*, 1994).

As described before, barley has basal PI levels in vegetative tissues. Some barley cultivars showed different susceptibility when are attacked by grasshopper (Weiel and Hapner, 1976). They suggested that the PI basal levels or their induction may affect insect choice for a host plant. Nevertheless, neither a direct evidence of PI induction by grasshopper damage nor deterrence toward the insect was proved.

Protease inhibitory activity induction by aphids has not been described yet. In our laboratory we are trying to characterize PI accumulation in barley infested by cereal aphids. Our preliminary results indicate that in infested barley leaves there is a two-fold increment of PI activity. This response is small compared to that found in solanaceous plants. We find that the PI peak activity is reached 48 hours after infestation with aphids. The main induced activity corresponds to a chymotrypsin inhibitor (unpublished results). Whether these inhibitors may have effects in digestion or the feeding behavior of the insects is yet to be determined. It is known that aphids can avoid deterrent compounds-rich organelles by-passing the vacuoles or by probing intercellularly (Dreyer and Campbell, 1987).

A possible wound-induced PI in barley leaves was discussed before (Kirsi and Mikola, 1977). The authors stated that, at least in barley, a signal mechanism different to that of dicots must be involved, since they found no PI induction. PIIF activity was also tested in barley and again a response similar to that in tomato was not found. Barley extracts, however, showed more PIIF activity on tomato plants than extracts from tomato leaves (McFarland and Ryan, 1974). Few attempts have been carried out to study the difference between dicots and monocots.

Nevertheless, PI induction has evolved as a defense mechanism, and it shows up regardless the injury-causing agent. Thus, PIs induced by one agent are present as a barrier to another pathogen or pest that attacks the plant after the induction of PIs has occurred. Then, PIs could serve as a mechanism of cross protection.

FUTURE CONSIDERATIONS

The production of proteinaceous inhibitors toward proteolytic enzymes has been named as an example of a 'primitive immune response' (Ryan, 1973). Thus, increasing protection of plants against pests resides in the same plant.

Members of the Bowman-Birk, Kunitz, cysteine, potato PI II, tomato PI I and cereal super family, have all been shown to increase resistance against insects or pathogens when expressed in transgenic plants. Because of great losses caused by pests and the ex-

pensive chemical treatment that crops require, one goal is the development of agronomically important crops with traits for more resistance using genetic engineering in plants. This constitutes now the main approach in the research done in this field. For example, in the experiments with the Bowman-Birk inhibitor from cowpea, soybean trypsin inhibitor and oryzacystatin, the bitten leaf area and insect survival were reduced down to 50% (Hilder *et al*, 1987; Boulter, 1993).

Perhaps better results may be obtained when using combined PI genes in the same crop. It is unlikely, however, to achieve 100% efficiency. Thus, an integrated pest management program must be performed for a given crop in which biological control, chemical insecticides and transgenic crops with PI genes or another insecticidal protein participate.

To introduce genes that code for proteins that are effective against a broad spectrum of pests, without affecting beneficial insects (*e.g.*, predators of pest populations and pollinators; Malone *et al*, 1995) or the metabolic functions of the transgenic host plant, genetic engineering may be used. Considering the increasing number of experiments with plants transformed with PI genes, it is essential to determine whether the exogenous PIs produced in these plants can also interfere with endogenous proteinases.

To select the most effective PI against a given insect, artificial bioassays must be carried out. Insects have evolved along with the development of synthetic insecticides. A mutation in the digestive enzyme of the insect is unlikely. Since PIs act at the catalytic site of the respective protease, it is difficult for insects to evolve a resistance mechanism in this highly conserved reactive site (Hoffmann *et al*, 1992).

As mentioned before, the specificity of an inhibitor may be changed through synthesis introducing few amino acids. This approach may bring advantages for using PIs in genetically engineered plants. Besides the conversion of a trypsin inhibitor into a chymotrypsin one, there are other examples, such as the production of chymotrypsin and elastase inhibitors from the *Cucurbita maxima* trypsin inhibitors (Kupryszewski *et al*, 1994). To get outstanding expression of PIs, some tissue specific and promoters sequences induced by wounding (Keil *et al*, 1990; Sanchez-Serrano *et al*, 1990; Ryan, 1992) or chemicals such as jasmonic and abscisic acids (Kim *et al*, 1992; Xu *et al*, 1993) are available.

During the last few years, considerable progress has been made in the understanding of plant defense mechanisms, as well as advances in the development of pest resistant crops. Whereas several PI genes have shown a marked potential for the improvement of plant defense, many of these inhibitors have not yet been tested against pathogens and other insects. It seems that this potential resource has just begun to be exploited.

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