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Protease inhibitor gene transfer as a tool for an ecological and sustainable plant defence strategy

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ABSTRACT

The plant defence strategy involving transfer of genes coding for enzyme inhibitors in a crop is based on the use of a natural mechanism activated by plants to defend themselves. Elicitors generated by mechanical wounding or by chemicals bind specific receptors on the plant cell membrane from where the expression of genes coding for inhibitors of proteases or amylase is activated through the octadecanoic acid pathway.

The engineering of protease inhibitor genes has been successful for many plant species of high commercial interest: tobacco, rice, poplar, tomato, lettuce, etc.

New protease inhibitor genes having peculiar and unique features have been identified on the genomes of several wild and cultivated *Cruciferae* and account for a new family recently defined as the MSI family.

The transfer of some of these genes has revealed significant effects on the defences of various plant species such as tobacco, oilseed rape and arabidopsis against lepidopteran insects and aphids.

Keywords: Plant defence strategy, agrochemicals, insect resistance, gene transfer, transgenic plants, Bt toxin, Proteinase inhibitor gene, Sustainable defence strategy.

Insect resistance: from chemicals to gene transfer

Losses of agricultural production due to pests and diseases have been estimated at 37% in Europe and worldwide. Worldwide, over 200 plant diseases are known to be transmitted by insects, mites, and nematodes.

Although the ability of insects to tolerate certain chemical treatments has been known since the 1890s and the first scientific articles dealing with insect resistance to insecticides in a systematic way appeared in around 1915(?), until now crops have been defended almost exclusively using chemicals. In the 1920s and 1930s, additional instances emerged in the field of insect resistance to

agrochemicals suggesting a growing contingent of economic entomologists to turn their research attention to these problems. At the same time, investigators began the study of resistance under the more readily controlled conditions of the laboratory using standardized insects such as the flour beetle and *Drosophila*. Despite the lack of a unified explanation for resistance, the majority of these researchers confirmed a previous initial hypothesis (Melandner, 1914; Quayle, 1922) that resistance resulted from a physiological adaptation of individual insects in a population that could be passed along genetically from one generation to the next.

Before the 1940s, chemical insecticides were based on inorganic substances such as arsenic, copper and sulphur, on petroleum products, and on derivatives of natural products such as nicotine from tobacco and pyrethrum from the chrysanthemum flower (De Ong, 1956). During the 1930s, several chemical companies began to investigate the pesticidal properties of various kinds of synthetic organic compounds. One of the first to be developed, and certainly the most well known, was DDT. The insecticidal properties of DDT were identified in 1939 by Paul Müller, a chemist with the J.R. Geigy Company of Basel, Switzerland. Following successful laboratory and field testing, Geigy began marketing DDT in 1941 under the trade name Gesarol. It was used primarily by Allied and German forces during World War II. However, in 1947, insect resistance to DDT was mentioned for the first time in the Geigy annual technical report entitled "Methods to Combat the DDT-Resistant Flies in Sweden", . Although several agricultural chemicals companies employed entomologists to conduct fundamental research on insect resistance beginning in the 1950s, the typical industrial strategy of developing novel chemical compounds remained unchanged up to the 1960s. Economic entomologists, on the other hand, gradually expanded their focus on chemical control of insects to include so-called biological methods that would hinder, if not prevent, the emergence of resistance in the field.

At present, crop protection relies predominantly on the use of environmentally toxic agrochemicals that are also deleterious to human health. Instead of using chemical pesticides on a large scale, other alternatives have been explored based on an interdisciplinary approach with system-oriented science and technology and are beneficial in terms of sustainable agriculture. Such sustainable systems will decrease inputs of energy and chemicals, and will not generate harmful outputs such as pesticide residues (Boulter, 1993).

Natural bio-control agents such as pathogens, predators, or parasites should be considered first of all. This method uses antagonists that follow various modes of action such as (i) competition for nutrients, (ii) competition for space, (iii) siderophore production, (iv) antibiosis, and (v) production of hydrolytic enzymes or other active substances. Examples of such biological control include, among others, the use of a non-pathogenic strain of *Ralstonia solanacearum* against its pathogenic

isoform; the *Vedalia* beetle, *Rodolia cardinalis* against the cottony-cushion scale pest and the egg-parasitoid *Trichogramma evanescens* as a potential biological control agent against the Indian mealmoth *Plodia interpunctella* (Benedict et al., 2003; Lyson, 2002; Prozell et al. 1995). The complex mechanism of action exerted by many such biocontrol agents gives them advantages over chemical pesticides, hampering the build up of resistance against them in the target pests (Gerhardson, 2002).

Secondly, various preventive pest control strategies employing crop rotation, intercropping, and cultivation of pest-resistant varieties of plants should be considered. They also involve the application of cultural control practices, such as the use of clean planting material, systematic trapping of adult insects (to control population build-up), and field sanitation (whereby residues that may form breeding grounds are removed).

Finally, transgenic insect-resistant crops expressing the insecticidal Bt toxins should be considered. Another insecticidal gene has been developed from the bacterium *Bacillus cereus*, known as the vegetative insecticidal protein ('vip ') gene, that is effective against corn rootworms (Benedict et al., 2003). These systems, all together, achieve the same objective: the necessity to enhance the resistance of plants to pests and pathogens through Integrated Pest Management (IPM) programmes that comprise a combination of control strategies including the judicious use of pesticides, crop rotation, field sanitation, and above all exploitation of inherently resistant plant varieties. These farming practices fall under the various headings of Chemical Control, Biological Control, Cultural Control, Mechanical Control, and Genetic Control.

Genetic control or the use of transgenic crops (Estruch et al., 1997) expressing foreign insecticidal genes could make a significant contribution to sustainable agriculture and thus is an important component of IPM.

A milestone in this field was the introduction of *Bacillus thuringiensis* (*Bt*) entomotoxic proteins into plants.

Bt toxin technology: pros and cons

At present, the main strategy to develop insect-resistant plants via genetic engineering is based on the use of Bt toxin genes. This has resulted in transgenic crops with a high resistance to specific insects. Current transgenic Bt crops expressing the Cry protein genes (crystal proteins or endotoxins) target key pests and also those resistant to conventional pesticides. Indeed, these proteins have several advantages such as a high specificity, a short life in the environment, and high and fast toxic activity (Benedict et al., 2003; Giband, 1998; Jouanin et al. 1998). Hence, it is not surprising that Bt makes up 98% of all biopesticides and represents the quasi exclusive source of pest-resistance genes for the development of transgenic plants. There are also significant

environmental benefits such as the absence of pesticide drift, the absence of residual pesticide in the soil, and the absence of effects on non-target species. Since the toxin is expressed throughout its tissues, the transgenic plant effectively controls root pests, stem and fruit borers, and sucking insects, unlike the conventional Bt sprays that could only protect the plant surface (Benedict et al., 2003; Brousseau et al., 1999).

However, there are limitations to 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). Also, the range of insects which can be controlled by Bt toxins is relatively narrow. Moreover, *Bt* toxins have a fairly complex mode of action. Many of the protoxin classes need to be proteolytically activated after solubilization in the guts of susceptible insects. The activated toxins then bind to receptor(s) on insect mid-gut epithelial cells and eventually integrate into the membrane where they create ion channels. Disruption of intracellular ionic homeostasis ensues leading eventually to cell death presumably by a colloid osmotic type mechanism (Knowles, 1994; Schnepf et al., 1998).

No Bt toxin with adequate aphid toxicity has been described and aphids continue to cause significant crop losses. Hence, it is necessary and desirable to develop safer and more effective transgenic alternatives to reduce crop losses. Improved and extended genetic crop resistance is usually seen as a foremost possibility in the current scenario of preventing agricultural losses due to insects and diseases. Another approach to slowing down insect growth is to use genes that encode for natural defensive compounds that are found abundantly in seeds, namely proteinase and α -amylase inhibitors.

In this paper we focus our attention on the use of resistant plants expressing proteinase inhibitors (PIs) against insects. There have been reported many examples of PIs active against certain insect species, both in vitro assays against insect gut proteases (Macedo et al., 2003; Wilhite et al., 2000) and in vivo artificial diet bioassays (Ashouri et al., 1998; Broadway et al., 1986; Franco et al., 2003).

Moreover, since PI genes are primary gene products, they are excellent candidates for engineering pest-resistance into plants (Boulter, 1993).

Plant proteinase inhibitor (PI) genes as a natural phytoprotection

Inhibitor genes of plant origin are particularly promising. This was first demonstrated by Hilder et al. (Hilder et al., 1987) by transferring trypsin inhibitor gene from *Vigna unguiculata* to tobacco, which conferred resistance to a wide range of insect pests including Lepidopterans, such as *Heliothis* and *Spodoptera*, Coleopterans, such as *Diabrotica* and *Anthonomous*, and Orthoptera,

such as Locusts. Following these early reports on the success of genetically engineered resistance in plants, several other PIs from various plant sources have been studied.

The availability of different genes from different plant sources is in itself an advantage as two or more genes can be transferred in combination (with different physiological targets) (Urwin et al., 1998). PIs are also reported to be active against nematodes, viral, bacterial, and fungal pathogens; thus, they may have a cumulative protective effect on plants. Further, there is no evidence that PIs have toxic or deleterious effects on mammals. These advantages make PIs an ideal choice for use in developing transgenic crops resistant to insect pests.

The inhibitors discovered so far have been found to be specific for each of the four mechanistic classes of proteolytic enzymes, and based on the active amino acid in their “reaction centre” [Belew et al., 1976] are classified as serine, cysteine, aspartic, and metallo-protease inhibitors. 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. Recently, all plant PIs have been catalogued in a database (<http://bighost.area.ba.cnr.it/PLANT-PIs/>), where their classification is mainly based on the protein primary structure. The database contains 10 families; nine of them are well known and correspond to classifications previously reported (De Leo et al., 2002).

Serine PIs 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. Serine PIs have been reported from a variety of plant sources (Odani et al., 1986; Antcheva et al., 1996; Haq et al., 2003; Hilder et al., 1989; Kort, 1980; Lin et al., 1991; Macedo et al., 1992; Mello et al., 2002; Negreiros et al., 1991; Pearce et al., 1982; Plunkett et al., 1982) and are the most-studied class of PI. Serine PIs are widespread in the plant kingdom, their physiological roles include the regulation of endogenous proteinases during seed dormancy, the reserve protein mobilization, and the protection against the proteolytic enzymes of parasites and insects. Moreover, they may also act as storage or reserve proteins.

Extensive research on PIs has provided a basic understanding of the mechanism of action that applies to most serine PI families and probably to the cysteine and aspartyl PI families as well. All serine inhibitor families from plants are competitive inhibitors of the protease that they inhibit with a similar standard mechanism as proposed by Laskowski and Kato (1980). Inhibition occurs as a consequence of binding of the active-site substrate-binding region of a proteinase to the corresponding substrate-like region (reactive site) on the surface of the inhibitor. The reactive site peptide bond after hydrolysis (i.e., in the modified inhibitor) acquires a newly formed carboxyl terminal residue designated as P1 and a newly formed amino terminal residue designated as P1'. The reactive site residue P1 generally corresponds to the specificity of the cognate enzyme. Thus,

inhibitors with P1 Lys and Arg tend to inhibit trypsin and trypsin-like enzymes, those with P1 Tyr, Phe, Trp (artificial only), Leu, and Met inhibit chymotrypsin and chymotrypsin-like enzymes, and those with P1 Ala and Ser inhibit elastase-like enzymes.

In response to feeding or wounding by insects, plants produce protease inhibitors which are usually active against endoproteases (Ryan 1990). The highest levels are found in very young leaves while older leaves have the lowest levels. As reported by Lawrence and Koundal (2002), current evidence suggests that the production of the inhibitors occurs via the octadecanoid (OD) pathway, which catalyzes the breakdown of linolenic acid and the formation of jasmonic acid (JA) to induce PI gene expression.

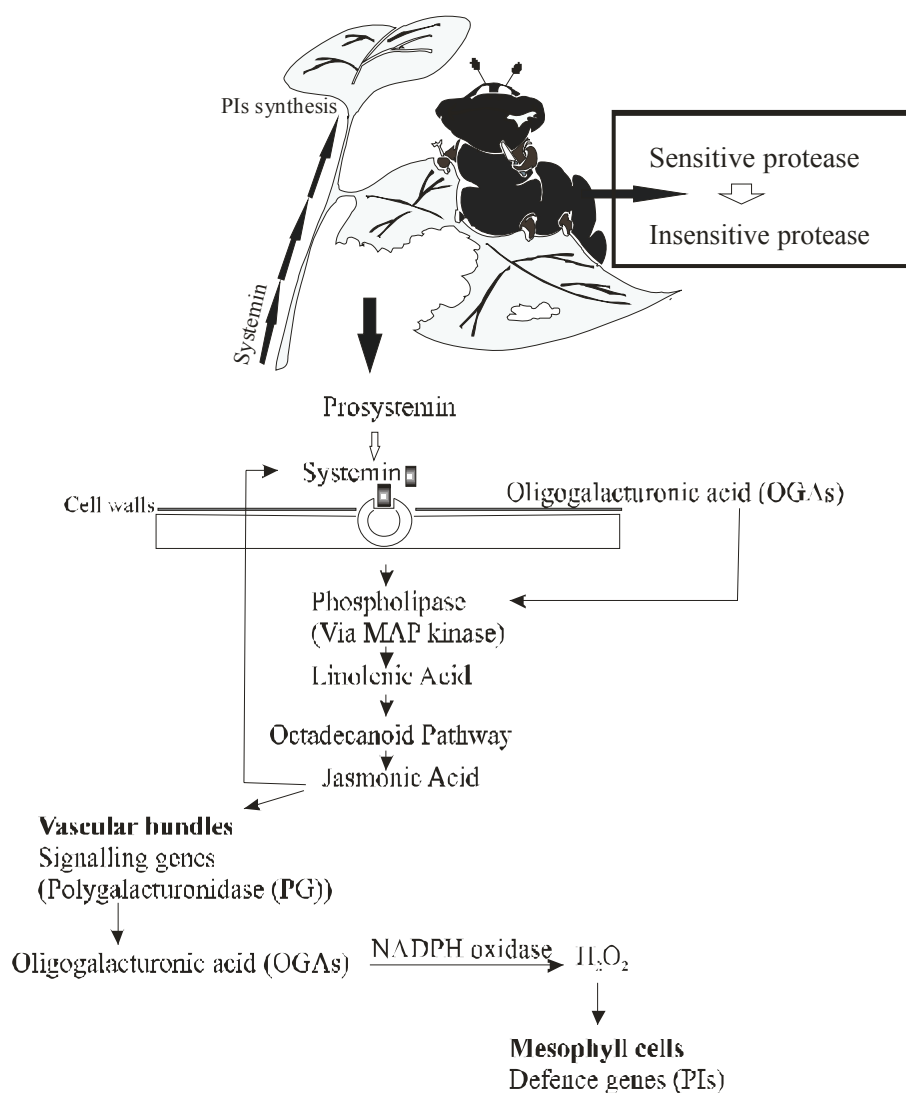


Figure 1. Schematic diagram of the signalling pathway necessary for local and systemic synthesis of PIs in plants by insect chewing. The schema shows also the insect adaptation due to PI ingestion.

There are differing views on the mechanism of development of resistance. Reese (1983) proposed the simple mechanism that growth rates were reduced due to reduced rates of proteolysis. This was later dismissed when Broadway and Duffey (1986) suggested that a feedback mechanism led(?leads) to the hyper-production of proteinases to compensate for the loss of activity, which in turn led to the depletion of essential amino acids and finally resulted in retarded growth rates. It was found that rats and chicks fed on low soya protein or STI extract developed a hyperactive pancreas and their intestines contained greater amounts of pancreatic enzymes including trypsin even though a large percentage was inhibited. So depression of growth was not due to blocking of proteolysis, but to a hyperactive pancreas. Nevertheless, the primary site of action of these inhibitors is the digestive system of insect larvae.

Since trypsin is involved in developmental processes such as moulting and synthesis of neuropeptides, trypsin inhibitors can disrupt these processes thereby retarding growth and development of the larvae (Lipke et al., 1954; Shukle et al., 1985; Steffens et al., 1978). In a study conducted by Marchetti et al. (2000). It was observed that larvae fed on transgenic plants expressing a Kunitz inhibitor gradually lost their turgor and became shrunken; hence it appears that food avoidance also has a dramatic effect on the water balance of the feeding larvae.

Since inhibitors block intestinal proteases, the first thing to consider is that PIs could cause a decrease in the absorption of amino acids and lack of growth in animals. However, studies on a range of experimental animals show that this is not the case. Although STI inhibits almost all trypsins in vitro, it does not cause poor growth in all animals. STI blocks trypsin leading to a pancreas feedback mechanism; the pancreas goes into hyper-production of pancreatic enzymes causing rapid depletion/loss of essential nutrients. However, it is only a serious problem in animals with a simple feedback/control of pancreas secretion. Humans and cattle have more sophisticated systems involving several other proteases, thereby eliminating doubts about growth limiting problems in mammals (Schulke and Murdoch, 1983). There is no evidence that PIs have toxic or deleterious effects on mammals. Rather, they are known to improve nutritional quality (Ryan et al., 1989). Many of these PIs are rich in cysteine and lysine, which contribute to enhanced nutritional quality. Large amounts of inhibitors are normally present in many raw foods of plant origin, but their anti-nutritional effect is drastically reduced with simple technological treatments such as cooking. The oral administration of small quantities of certain inhibitors (e.g., soybean Bowman-Birk) has a protective role against carcinogenesis in the oesophagus, duodenum, and colon (St Clair et al., 1980; Kennedy 1994).

For efficient management of pest control through PI transgenes, it is imperative to know the type of enzymes present in the guts of insects and pests. The two major proteinase classes in the digestive

systems of phytophagous insects are the serine and cysteine proteinases. Many Lepidopterans have serine proteinases as the major digestive enzymes, whereas Coleopteran species have a wider range of dominant gut proteinases (Boulter 1993; Christeller et al., 1992; Johnston et al., 1995; Johnston et al., 1991; Jongsma et al., 1996; Purcell et al., 1992).

Genetic Engineering: some aspects

Plant genetic transformation is a potential tool to understand genome regulation and to carry out the manipulation of genes that could otherwise not be done by classic breeding techniques. Using plant genetic engineering it is now possible to address the relationships between PIs and the growth, development, and survival of insect species in transgenic plants. Aspects of inhibitor specificity, insect proteinase specificity, physiological effects of proteinase inhibitor complexes or feedback mechanisms in insect digestion, and the roles of combined or synergistic effects of defensive genes can be assessed (McManus et al., 1994). Further, transformation of plant genomes with PI-encoding cDNA clones appears attractive not only for the control of plant pests and pathogens, but also as a means to produce PIs, useful in alternative systems, and the use of plants as factories for the production of heterologous proteins (Sardana et al., 1998).

The general methods of gene transfer commonly employed are:

- (1) The *Agrobacterium tumefaciens* mediated gene transfer method (Lee et al., 1999; Hinchey et al., 1988; Melo et al., 2003; Raineri et al., 1990).
- (2) PEG-mediated direct gene transfer to protoplasts of several cultivars (Chair et al., 1996; (?)Chair et al., 1996; Hosoyama et al., 1994).
- (3) Electroporation-mediated DNA delivery (Dillen et al., 1995).
- (4) Microprojectile-mediated gene transfer (biolistic method) (Finer et al., 1991; Genga et al., 1991).

Control and regulation of transgene expression are required for efficient gene transfer in transgenic plants. This regulation can occur during different steps of gene expression, but more particularly during transcription, and promoters ensure this control. Promoters with spatial and temporal expression patterns need to be studied to investigate and ultimately manipulate responses to biotic and abiotic stresses (Gaddour et al., 2001). Selectable “marker genes” and efficient promoters are also introduced alongside the gene to identify transformed plant cells from untransformed ones and to ensure efficient transcription of the gene (Rothstein et al., 1987; Schuler et al., 1998). Conventionally “antibiotic-resistance” marker genes have been used; but due to rising concerns about their use, they are now being replaced with herbicide-tolerance genes (e.g., chlorsulphuron) that can eventually be bred away.

PI genes in combat against insects and pests: the success story

Following several mechanistic studies on transgenic proteinase inhibitors, it was found that serine PIs are effective against Lepidoptera (Yeh et al., 1997; Lecardonnel et al., 1999) whereas cysteine PIs are effective against some Coleoptera (Lecardonnel et al., 1999; Leplé et al., 1995). Transgenic crops, including rice, expressing foreign PIs, show reduced predation by their natural pests. Table 1 shows some transgenic plants that have been genetically transformed to include proteinase inhibitor genes and their efficiency against natural pests, nematodes, and other pathogens of the host plants.

Table 1: Transgenic plants carrying proteinase inhibitor genes in defence against insects and pests

Proteinase inhibitor genes	Transformed plant	Defence against	Reference
Cowpea trypsin inhibitor (CpTI)	Tobacco	<i>Heliothis virescens</i>	(Gatehouse et al., 1993 ; Hilder et al., 1987)
	Cotton	<i>Spodoptera litura</i>	(Sane et al., 1997)
		<i>Helicoverpa armigera</i>	(Li et al., 1998)
	Rice	<i>Chilo suppressalis</i> and <i>Sesamia inferens</i>	(Xu et al., 1996)
	Potato	<i>Lacanobia oleracae</i>	(Gatehouse et al., 1997)
	Strawberry	<i>Otiorynchus sulcatus</i> F.	(Graham et al., 1997)
	Cabbage	<i>P.rapae</i>	(Fang et al., 1997)
Pigeon pea	<i>Helicoverpa armigera</i>	(Lawrence et al., 2001)	
Soybean (Kunitz) trypsin inhibitor	Tobacco	<i>Spodoptera litura</i>	(Mc Manus et al., 1999)
	Rice	<i>Nilaparavata lugens</i> Stal	(Lee et al., 1999)
Soybean Kunitz, C-II and PI-IV inhibitor	Potato/tobacco	<i>Spodoptera littoralis</i>	(Marchetti et al., 2000)
Tomato proteinase inhibitors I and II	Tobacco	<i>Manduca sexta</i> larvae	(Johnson et al., 1989)
Potato inhibitor II gene	Tobacco	<i>Chrysodeisus eriosoma</i>	(McManus et al., 1999)
	Rice	<i>Sesamia inferens</i>	(Duan et al., 1996)
Sweet potato (<i>Ipomea batatas</i>) trypsin inhibitor	Tobacco	<i>Spodoptera litura</i>	(Yeh et al., 1997)

Trypsin inhibitor from barley (CMe)	Indica and japonica rice Wheat	<i>Sitophilus oryzae</i> <i>Spodoptera littoralis</i>	(Alfonso-Rubi et al., 2003) (Alpeteter et al., 1999)
Mustard trypsin inhibitor-2	Tobacco Oilseed rape Arabidopsis Oilseed rape	<i>Spodoptera littoralis</i> <i>Plutella xylostella</i> <i>Mamestra brassicae</i> <i>Pterostichus madidus</i>	(De Leo et al., 2002) (De Leo et al., 2001) (De Leo et al., 2001) (Ferry et al., 1005)
Oryzacystatin I	Poplar Potato Oilseed rape	<i>Chrysomela tremulae</i> <i>Myzus persicae</i> <i>Leptinotarsa decemlineata</i> <i>Ceutorynchus assimilis</i> <i>Myzus persicae</i>	(Leplè et al., 1995) (Gatehouse et al., 1996) (Lecardonnell et al., 1999) (Jouanin et al., 1998) (Rahbè et al., 2003)
Corn cystatin	Rice	<i>Sitophilus zeamais</i>	(Irie et al., 1996)
<i>Nicotiana glauca</i> protease inhibitor	Tobacco Pea	<i>Helicoverpa punctigera</i> <i>Plutella xylostella</i>	(Heath et al., 1997) (Charity et al., 1999)
<i>Arabidopsis thaliana</i> cysteine proteinase inhibitor	White poplar (<i>Populus alba</i> L.)	<i>Chrysomela populi</i>	(Delledonne et al., 2001)

Although in most cases transformed crops show a significant increase in resistance to specific insects, in other cases, depending in particular on the PI gene transferred and on the insects attacking that species, the so called “adaptation effect” takes place. In these cases, new “insensitive proteases” are expressed and released in the gut of insects feeding on plants transformed by a specific PI gene, so nullifying the protective effect of the gene. To overcome PI resistance of “insensitive proteases” it is now necessary to further develop PIs that are broadly active against all proteases that insects use for digestion. The objective is achieved by:(1) selecting second generation protease inhibitors (novel PIs) from novel sources (unrelated organisms, synthetic libraries or insect haemolymph) with maximum effectiveness against the selected target pests; introducing novel PIs into important crop plants; and demonstrating, in the field or greenhouse, that a substantial

reduction in the application of insecticides can be achieved. (2) Use of bi-functional α -amylase/trypsin inhibitors is of particular practical interest since transgenic plants expressing a molecule which inhibited both amylases and proteases of pests would be highly protected. The presence of both the activities would also help to minimize the likelihood of the appearance of resistant pest strains. (3) Another approach may be to express two or more PIs as a fusion protein. Expression of a cystatin and a serine PI in this way has been found to be successful against certain nematode pathogens (Urwin et al., 1998). (4) Studies on the insecticidal activity of transgenic tobacco plants expressing both Bt insecticidal protein and cowpea trypsin inhibitor (CpTI) genes against cotton bollworm (*Helicoverpa armigera*) revealed that it was more effective compared to transgenic tobacco expressing the Bt insecticidal protein gene alone. In addition to the enhancement of insecticidal efficacy, insect adaptation to transgenic Bt crops is also delayed (Fan et al., 1999).

The MSI family: a success story only at the beginning

One of the major priorities in the use of transgenic plants is the evaluation of risks concerning human health and the environment. The strategy of transferring PI genes into plants to increase insect resistance should not, as explained in a previous Section, have effects on human health due to the specific target of these proteins. The risk to the environment is uncertain and it may be correlated to the level of PI expressed in plants and to the insect targets. As our group has demonstrated, the effectiveness of a PI (the MTI-2 protein) is related to the dose of inhibitor the insects ingest (REF). The gene chosen for these experiment was the one coding for the MTI-2 protein. The Mustard Trypsin Inhibitor-2 (MTI-2), was isolated from seeds of *Sinapis alba* and inhibits trypsin to a relevant extent (Menegatti, et al., 1992). The gene was detected on the genomic DNA and sequenced. It is discontinuous with a single intron located upstream of the region encoding the mature protein (Ceci, et al., 1995). The *mti-2* gene encodes a 99 amino acid precursor having 30 and six amino acids at the amino- and carboxy-termini respectively, neither of which are present in the mature protein. The transfer of the *mti-2* gene into crop and model plants gave interesting results: when expressed in tobacco, arabidopsis and rapeseed, MTI-2 increased plant defences against lepidopteran insects such as *Plutella xylostella*, *Spodoptera littoralis* and *Mamestra brassicae* (De Leo et al., 1998; De Leo et al. 2001). Interestingly, a threshold effect has been detected using a short term bioassay. Above a specific threshold, which must be evaluated case by case, insects die, but surviving larvae synthesize new digestive proteinases (De Leo et al., 1998). This effect had been demonstrated by performing two week bioassays on first instar larvae of *S.littoralis* (Lepidoptera) fed on high and low transgenic tobacco plants. In these experiments a relevant number of larvae were not able to survive after 15 days of treatment, and therefore were not able to reach the pupal stage. It has been also demonstrated that in oilseed rape and tobacco

expressing MTI-2 at low levels, no effect is evident on *S. littoralis* larvae or on other insects such as *P. xylostella* and *M. brassicae* fed on the same plants (De Leo et al. 1998, 2001).

The most interesting and new result obtained with *S. littoralis* larvae fed on tobacco plants expressing the *mti-2* gene has been described in De Leo and Gallerani (2002). It has been shown that the number of eggs per female decreased in *S. littoralis* fed on MTI-2 transgenic plants compared to controls. The effect was significant for plants expressing high levels of MTI-2, whereas only a tendency to reduction was evident for plants expressing low MTI-2 levels. These data indicate that females feeding on MTI-2 foliage were not equally competent sexually. A further interesting result was obtained by measuring the protease contents and their activities in the guts of second and third instar larvae fed on transgenic leaves. Since no differences occurred in the protease and activity patterns between larvae fed on controls or on plants expressing low and high MTI-2 levels, it can be concluded that those genetic traits were not transferred after the first generation. The results of experiments described in De Leo and Gallerani (2002) add more relevant information to those presented in the previous work (De Leo et al., 1998) concerning the strategy of increasing tobacco plant defences by transferring a protease inhibitor gene. In a field where transgenic tobacco plants expressing the MTI-2 inhibitor were cultivated at levels higher than the threshold previously established (De Leo et al., 1998), larval populations of different ages would be subjected to different effects. While the younger larvae (L1) would develop with significant mortality within the first 10–15 days, the other ones (L2) would reach the pupal stage but with a lowered fertility. Moreover, despite some of them adapting to the presence of the inhibitors, they would not transfer this trait to their progeny. The two effects would have a relevant consequence on the insect levels in the field, which would decrease over time. Therefore the pest control method based on the use of this PI would have a mild and reversible effect on the environment because the presence of insects would be restored as soon as the cultivation of that transgenic plant in that field was stopped.

The *mti-2* gene is the former inhibitor isolated from *Cruciferae* belonging to the MSI family described, for the first time, in the most recent classification of protease inhibitors proposed so far by De Leo et al. (2002). At the moment, the MSI family includes six members, all of them belonging to the *Cruciferae* family and having high similarity in their primary sequence. In particular, two inhibitors have been isolated with similar peculiar characteristics (Menegatti et al. 1992; Ceciliani et al., 1994). One is the MTI-2 protein already described, the second is the RTI-III (Rapeseed Trypsin Inhibitor-III) highly similar to MTI-2 (Ceciliani et al, 1994) purified from oil rape (*Brassica napus* var. *oleifera*) seeds. This protein inhibits trypsin and chymotrypsin to the same extent as the mustard inhibitor.

Genes coding for MSI family members have also been identified in the *Arabidopsis thaliana* genome (atti genes) and the putative precursor form of one of them has been expressed in *Escherichia coli* (Zhao et al., 2002). ATTI, MTI-2, and RTI are highly homologous low-molecular-mass proteins (7kDa), impairing trypsin rather than chymotrypsin action.

The physiological functions of some components of the MSI family available at the moment as native or recombinant proteins are very difficult to establish even if not all of them have been fully explored. Because RTI-III, MTI-2 and related iso-inhibitors (see below) are found in seeds as are other protease inhibitors, it is possible to speculate that they may regulate endogenous proteinases during seed dormancy or may serve as a storage source of protein (Ryan, 1990). The function of MTI-2 under certain circumstances has been attributed to plant defence (De Leo et al., 2001, De Leo and Gallerani 2002). Indeed, as far as the *mti-2* expression is concerned, northern blot experiments (Ceci et al. 1995, De Leo et al., 2001) demonstrate that *mti-2* is transcribed under developmental conditions (i.e. during the late stages(?) of seed maturation). During this period of time, the expression of *mti-2* is very likely to lead to the accumulation of the mature protein needed for the inhibition of endogenous proteinases. Moreover, the detailed analysis reported by De Leo et al. (2001) shows that the *mti-2* gene is also expressed in response to mechanical and chemical induction, suggesting that synthesis of the MTI-2 protein in the aerial part of the plant could be related to the activation of defence mechanism(s).

Moreover, the *mti-2* gene and several mutants obtained by site directed mutagenesis have been expressed in *Pichia pastoris* and the specificity of the MTI-2 reactive site (R20-I21) demonstrated (Volpicella, et al., 2000;. Volpicella et al., 2001). Other mutants with different affinities against trypsin and chymotrypsin have also been produced using the phage display approach (Ceci, et al. 2003). Among those, a mutant specifically obtained by selection against chymotrypsin proved significantly effective in reducing the life span and vitality of the aphid species *Acyrtosiphon pisum*.

Conclusions

With the development of disease- resistant and insect-resistant crops, genetic engineering has addressed at least some of the environmental problems associated with conventional agriculture. With the increasing demand for food for the burgeoning human population world-wide and decreasing cultivable land, it appears that plant genetic engineering has to be adopted for maximum benefits with minimum input. Losses due to pests have to be minimized and development of transgenic, insect- and pest-resistant crop varieties through PI genes will make a promising contribution towards maximizing yields. However, agricultural biotechnologies are anchored to a scientific paradigm rooted in experimental biology, whereas sustainable agriculture rests on a

biological paradigm that is best described as ecological. Nevertheless, there are still a lot of promising possibilities for pest control through insecticidal genes that need to be explored and prudently tapped for their implementation in IPM programmes.

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