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review

Proteinase Inhibitors Offer the Possibility of Producing Disease Resistant Transgenic Potatoes*

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Summary

Proteinase inhibitors are not only important regulators of plant endogenous proteases, but also contributors to the plant defence against insects and pathogenic microorganisms. They are normally present in plant storage organs, but after attack by predators, their synthesis is induced throughout the plant. Proteinase inhibitors inhibit a broad range of insect digestive proteinases and, as a consequence, reduce the growth rate and increase the mortality rate of several insect larvae. A modern biotechnological approach offers the possibility of enhancing the natural defence of crop plants by the transfer of genes encoding an appropriate spectrum of proteinase inhibitors.

Key words: potato proteinase inhibitors, insect digestive proteinases, transgenic plants, protease inhibitor gene expression

Introduction

Plants grown in the soil are inevitably exposed to sometimes very hostile environments. In order to survive they have to respond very quickly to the external stimuli, such as attack by predators, pathogenic microorganisms or some forms of physical stress (low temperatures, drought, *etc.*). The interesting feature is that these environmental signals also serve as signals in developmental regulation. Many genes that are expressed at specific times of vegetative and reproductive growth can also be activated at other times in response to adverse environmental stimuli. Besides constitutive resistance mechanisms, which are present before the attack of pests, plants have also evolved inducible mechanisms which lead to the activation of defence genes and the synthesis of defense-related proteins. Based on their role in defence response these proteins have been divided into three groups *(1)*. The proteins of the first group include structural proteins and a number of enzymes that affect the defence status of the plant by strengthening, repairing or altering the cell wall. Proteins of the second class are so called pathogenesis related proteins. These are low-molecular mass proteins which accumulate extracellularly and are resistant to proteolysis and acidic pH. The third class contains proteins associated with deterrence and antimicrobial activity, including enzymes involved in the synthesis of oxydised phenolics, tannins and phytoalexins, toxic proteins and inhibitors of proteinases and amylases. In this short review attention will be focused on proteinase inhibitors, with special emphasis on those from potato, their role in plant defence response as well as on the possibilities of transfer of their genes in order to enhance the natural defense of many crop plants which have been weakened through breeding.

Proteinase Inhibitors in Plants

Proteinase inhibitors were discovered over 60 years ago. Interest in these proteins has expanded from earlier research on their effect on human food *(2)* to more recent interest in their possible role in plant metabolism *(3)* and plant defence mechanisms. In addition, their nu-

* Dedicated to Professor Pavao Mildner for his 80th birdthday

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tritional significance in food, in particular the possible contribution to the prevention of various types of cancer, has again stimulated investigations of native, modified and synthetic inhibitors *(4)*. Inhibitory proteins are usually found concentrated in seeds and tubers, particularly in the families *Poaceae, Fabaceae and Solanaceae.*

Proteinase inhibitors are classified into four mechanistic classes according to proteinases they inhibit: inhibitors of serine, cysteine, aspartic and metallo proteinases *(5)*.

Inhibitors of serine proteinases

Inhibitors of serine proteinases have been extensively studied. They have been isolated from plant storage tissues such as seeds and tubers where they often account for several percent of the total protein. Based on their sequence data, position of the reactive sites and the position of disulphide bridges they are subdivided into eight families *(6)*, as shown in Table 1.

Table 1. Families of plant serine proteinase inhibitors

Soybean trypsin inhibitor (Kunitz) family
Soybean trypsin inhibitor (Bowman-Birk) family
Potato inhibitor I family
Potato inhibitor II family
Barley trypsin inhibitor family
Squash inhibitor family *(7)*
Ragi α-amylase/trypsin inhibitor family *(8)*
Serpin family *(9)*

An extensive review lists newly discovered inhibitors from various plant sources with new inhibitory and other functions *(3)*.

The mechanism of action of these inhibitors involves a reactive site region which contacts the active site of the enzyme and forms a stable complex with a very high equilibrium constant for association, similar to an enzyme – substrate complex in which the enzyme cannot complete the hydrolysis of the peptide bond. The specificity of the inhibitor is determined by the amino acid residue at the P1 site. Besides single headed inhibitors, double headed inhibitors exist that can inhibit two proteinases of the same or different mechanistic class simultaneously and independently.

Inhibitors of cysteine proteinases

Within the last 15 years the potent reversible inhibitors of cysteine proteinases (called cystatins) have been found in animals, plants and microorganisms. They form a super family of cystatins and are further divided into three families: stefins, cystatins and kininogens *(10)*. Recently a new group of cysteine proteinase inhibitors has been discovered that is structurally different from cystatins. Their characteristic feature is that they contain one or more thyroglobulin type–1 domains, and were therefore named thyropins *(11)*. Two inhibitors of the thyropin family have been isolated and their structure determined: equistatin from sea anemone *(12)* and ECI from the egg of chum salmon *(13)*. Cysteine proteinase inhibitors have been isolated from various plants, including rice *(14)*, corn *(15)*, soybean *(16)*, sunflower *(17)*, carrot *(18)* and greater celandine *(19)*. These protein inhibitors show a high degree of sequence homology to the cystatin family, but unlike the stefins they do not contain disulphide bonds. It was proposed therefore that they should be classified as the phytocystatin family *(14)*. This proposal was further supported by the finding that the gene structure of one of phytocystatins is different from that of animal cystatins *(20)*. The inhibitory mechanism of cystatins involves close contacts between enzyme and a hydrophobic tripartite wedge of the inhibitor, complementary to the active site. The inhibitor binds to the enzyme in a substrate-like manner but is not cleaved. The binding sites of stefins and cystatins consists of two β-hairpin loops, a highly conserved QVVXG region and the N-terminal sequence.

Inhibitors of aspartic proteinases

In contrast to serine and cysteine proteinase inhibitors, very few inhibitors of aspartic proteinases of plant origin are known. Cathepsin D isoinhibitors have been isolated from potato tuber and studied in details. Their primary structures show a similarity to the Kunitz soybean trypsin inhibitor (STI) family *(21,22)*. In addition, cDNA sequences of potato cathepsin D inhibitor homologues were determined and their deduced amino acid sequences showed more than 70 % identity with the sequences of isolated isoinhibitors *(23–25)*. The primary structure of the aspartic proteinase inhibitor gene has been determined *(26)* and the existence of a multigene family in potato and also in other solanaceous species was confirmed *(27)*. Aspartic proteinase inhibitors from potato inhibit trypsin as well as cathepsin D. The presumed active centre responsible for trypsin inhibition (Arg 67–Phe 68) is present in all homologues. They also possess an extra region of seven or eight amino acids at the C-terminal part of the molecule which might be involved in cathepsin D inhibition *(28)*. Recently an inhibitor of pepsin from squash was isolated with no sequence similarity to potato aspartic proteinase inhibitors *(29)*

Inhibitors of metalloproteinases

Very few plant inhibitors have been found which specifically inhibit pancreatic carboxypeptidases A and B and these were isolated from potato and tomato *(30)*.

Proteinase Inhibitors in Potato

Potato (*Solanum tuberosum* L.) is the most important non-cereal world food crop. Unfortunately, it is prone to various diseases caused by microorganisms and suffers from attack by insects and nematodes. Therefore intensive potato production in many parts of the world relies heavily on the extensive applications of number of agrochemicals. The use of pesticides results in the development of pathogen resistance, occurrence of secondary pests and in environmental pollution. Potato breeding for disease resistance, while retaining all desirable agronomic characteristics of the crop, is very difficult. The modern biotechnological approach using gene transfer has been tested in some crop plants in an attempt to increase the natural defense and suggests the possible use of such an approach also in potato.

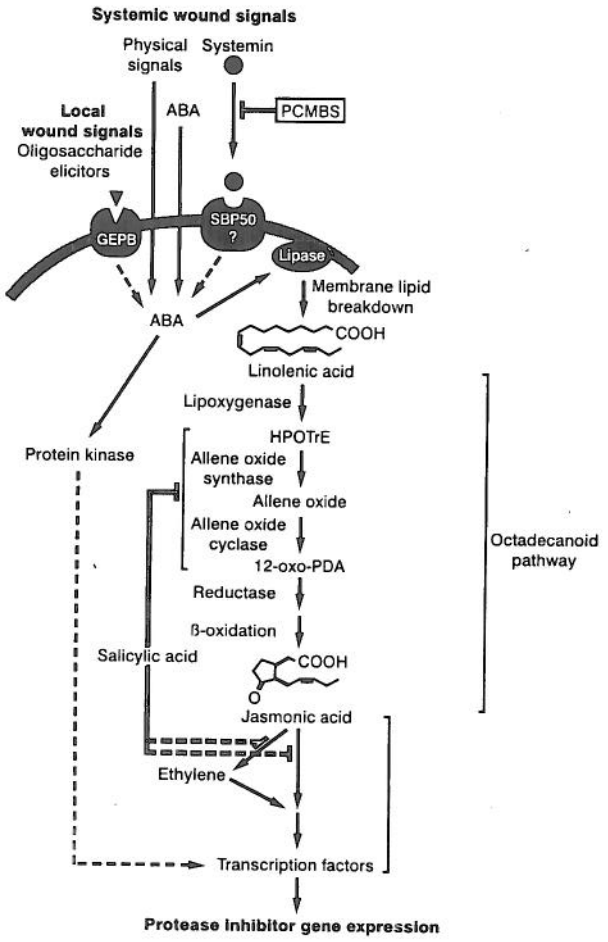

Fig. 1. Proposed scheme for the signalling that leads to the expression of protease inhibitor genes (modified from 46). Solid lines show the portions of the pathway proposed from direct evidence, broken lines show inferred pathways. Open bars illustrate the effect of inhibitors.
PCMBS, p-chloromercuribenzene sulfonic acid; ABA, abscisic acid; GEBP, β-glucan-elicitor-binding protein; SBP50, systemin-binding protein of 50kDa; HPOTrE, 13(S)-hydroperoxylinolenic acid; 12-oxo-PDA, 12-oxo-phytodienoic acid.

Potato tuber is a rich source of proteinase inhibitors which have been intensively investigated at the gene and protein level. Structural and mechanistic studies have shown that they cannot easily be assigned to only one of the four mechanistic classes. The most representative members of serine proteinase inhibitors are potato inhibitor I, which inhibits chymotrypsin *(31)* and potato inhibitor II, an inhibitor of chymotrypsin and trypsin *(32)*. Two low-molecular weight inhibitors of trypsin and chymotrypsin (polypeptides of 51 and 52 aminoacids) with a high sequence similarity to potato inhibitor II have also been found in significant quantities in potato tubers *(33)*.

Several isoinhibitors of cysteine proteinases of Mr = 20–24 kD have been isolated from potato and found to be structurally different from cystatins *(34)*. They are potent inhibitors of animal lysosomal cysteine proteinases, but some of them exhibit a weak inhibitory activity against trypsin and chymotrypsin *(35)*. Their amino acid sequences revealed that they are structurally related to the Kunitz type soybean trypsin inhibitor family *(36, 37)*. Further studies at the cDNA level showed that the inhibitors within this family can be divided into three major homology groups *(38)*. In the first and second group are inhibitors of aspartic and serine/aspartic proteinases *(21,23,25,26)*, whereas in the third group are inhibitors of serine, cysteine and serine/cysteine proteinases *(37)*. The superfamily of potato type of STI would thus comprise proteins inhibiting serine proteinases (trypsin and subtilisin family), aspartic proteinase (cathepsin D), cysteine proteinases (inhibitors of the papain family) and proteins with some other activity or function *(39)*. The mechanism of inhibitory action of these inhibitors has not yet been clarified.

Protein crystals found in potato tuber cells were shown to consist of a single 85 kD protein which inhibits cysteine proteinase. The inhibitor was named multicystatin because it consists of eight closely related domains, showing homology to the cystatin family *(40)*. It can bind eight papain molecules simultaneously.

Proteinase Inhibitors and Plant Defence

The accumulation of proteinase inhibitors in seeds and tubers supports their predicted role as defense related proteins, because this stage of the plant's life cycle should be highly protected in order to ensure reproduction and survival of the species. There are only a few data concerning the distribution of proteinase inhibitors in vegetative tissues. In the normal field grown potato plants the expression of the first two subgroups of potato Kunitz-type inhibitors genes was not detected or only detected at very low levels in stems, mature leaves and roots *(38)*. Potato inhibitor I and II were detected by immunogold labelling in the cell vacuoles of transgenic tomato plant, and some of them were secreted into the cell walls of root tips *(41)*. Using the same experimental approach, one of the aspartic proteinase inhibitors was found in smaller amounts in the cytoplasm, sometimes associated with the Golgi apparatus, and in the intercellular space of potato root cells of a plant, grown under non-inducible conditions *(37)*. This would suggest that they might be transported out of the cell by Golgi vesicles.

Mechanical wounding

Physical injury caused by the attack of herbivorous insects or pathogens drastically alters the plant's pattern of gene expression. As a result a number of proteins are being synthesised aimed mainly at wound healing, prevention of pathogen invasion and retardation of insect growth and development. The activation of genes can occur in an area close to the wound site or can also occur systemically in distal undamaged tissue. Proteinase inhibitors are among the most intensively studied proteins that accumulate not only in wounded leaves but systemically throughout the plant *(42)*. The best studied examples are potato and tomato proteinase inhibitor II (*pin 2*) gene families. The proteins of these family are constitutively expressed in potato tubers and in the early stages of floral development. On wounding (me-

chanical damage or herbivore feeding) *pin2* mRNA accumulates in potato and tomato plants. There is a short delay in *pin* 2 mRNA accumulation in non damaged leaves compared to the wounded ones *(43)*.

The local and systemic regulation of proteinase inhibitor gene expression is not limited to potato I and II families. Induction of aspartic proteinase inhibitor in wounded potato leaves was observed two days after injury *(44)*. and induction of multicystatin was observed three days after wounding *(45)*.

Proteinase inhibitor genes can also be activated during attack by pathogenic microorganisms, by oligosaccharide fragments (elicitors) generated from both plant and fungal cell wall. Induction of proteinase inhibitor genes is mediated by plant derived chemicals, hydraulic signals and electrical signals. Several models for the signal transduction pathways have been proposed (reviewed in *46*). The pathway shown in Fig. 1 starts with the interaction of systemic signals released as a consequence of mechanical wounding, i.e. polypeptide systemin, or localized signals (oligosaccharides), with plasma membrane receptors. As a result, a lipase is activated that facilitates the release of linolenic acid from membrane lipids. Linolenic acid is then converted via several intermediates to jasmonic acid which, as it is proposed, interacts with a receptor and induces the expression of proteinase inhibitor genes. Exogenously applied jasmonic acid and its methyl ester can activate the synthesis of proteinase inhibitor in leaves more powerfully than does wounding. Several plasma membrane receptors are being implicated in the current models. A receptor that binds to fungal oligosaccharide elicitors has been isolated from soybean *(47)*, as well as systemin binding protein from tomato leaves *(48)*. Abscisic acid (ABA) is also a local and systemic signalling molecule involved in signal transduction. It probably functions somewhere between systemin and the linolenic acid pathway but it is not known whether ABA and jasmonic acid pathways interact *(49)*. Another molecule involved in the transduction pathway is ethylene, a gaseous plant hormone. On wounding ethylene regulates the level of endogenous jasmonic acid, whereas exogenous application of jasmonic acid induces ethylene biosynthesis which is required to induce *pin* gene expression. Two processes contribute to the wound induced increase in jasmonic acid but only one is ethylene dependent *(50)*. Among known inhibitors which block the signal transduction pathway is *p*-chloromercuribenzene sulfonic acid which blocks the systemin translocation through phloem and reduces proteinase inhibitor gene expression. Some inhibitors of lipoxygenase (salicylic acid) block the conversion of linolenic acid to jasmonic acid eliminating the wound- induced expression of proteinase inhibitors *(46)*.

Digestive proteinases of insects

Digestive enzymes from many insects have been studied and an extensive review was published recently *(51)*. Proteinases are essential enzymes for insect growth and development. Trypsin-like enzyme activities have been reported in most of the insect species examined, except for *Hemiptera*. The distribution of the chymotrypsin-like activities seems to be similar to that of trypsin. It was however low in the midgut of *Lepidoptera*. Cysteine proteinases are found in *Hemiptera* and *Coleoptera*. Aspartic proteinases have been detected in the larval midguts of *Coleoptera* and possibly *Diptera*. A detailed study of digestive proteinases from western corn rootworm larvae showed that at least 15 distinct proteolytic activities were detectable in the SDS gels *(52)*. An effective defence mechanism therefore depends on disruption of protein digestion in the insect and, as we have seen particularly in potato, there is a broad spectrum of proteinase inhibitors that are inhibitory to all classes of proteinases. In spite of this, potato is still a very vulnerable crop plant.

Transgenic plants containing proteinase inhibitor genes for increased insect resistance

Several attempts have been made to increase resistance in plants to pathogen and insect attack. Modified genes from *Bacillus thuringiensis* toxins have been introduced into tobacco *(53)* and cotton (54). Transgenic plants synthesised insecticidal proteins in sufficient amounts to protect them from feeding damage by tobacco hornworm and cotton bollworm. Transgenic plants that express tobacco mosaic virus coat protein gene delayed the disease and 10–60 % of transgenic plants did not develop symptoms during the duration of experiments *(55)*.

In view of their role as natural defence proteins, proteinase inhibitors have the potential to enhance resistance against insects in transgenic plants that have been transformed with their corresponding genes. This very promising approach has been used to produce transgenic tobacco plants overexpressing a trypsin inhibitor from the cowpea, transgenic tobacco harbouring a potato proteinase inhibitor II gene, corn cystatin gene, and others (reviewed in *56*), but the results have not always been up to the expectation. The protective effects have been limited and transient. As already mentioned, insects use multiple proteases for protein degradation, therefore the introduction of genes expressing different inhibitors with high inhibitory specificity would be needed.

Furthermore, it has been discovered recently that the insect *Spodoptera exigua* can adapt to the presence of either endogenous or transgenic serine proteinase inhibitors by the induction of new digestive proteinases insensitive to the inhibitors present in tobacco plant *(57)*. There are various possibilities for combatting these proteinases *(58)*. In one approach, various cysteine proteinase inhibitors of animal and plant origin were tested for the inhibition of digestive proteinases in the gut of Colorado potato beetle, feeding on potato plant which expressed high levels of endogenous proteinase inhibitors *(59)*. It was found that cystatins were poor inhibitors of induced digestive proteinases when tested *in vitro*. The strongest inhibition was obtained by equistatin, a thyropin type inhibitor from the sea anemone.

Conclusions and Perspectives

The effectiveness of proteinase inhibitors in plant resistance against insect attack depends on the ability to block the digestive proteases. Therefore, inhibitors must

be expressed at high enough concentrations and possess the necessary specificity toward all target insect proteases. Inhibitors can be selected from various plant and animal sources or even from the target insects where they are an essential physiological regulatory component for gut and tissue proteinases. In order to achieve a tight and stable complex between proteinase and inhibitor it is possible to improve the interactions by genetic engineering using computer modelling or selection based on phage display. Instead of producing a transgenic plant that would express a mixture of inhibitors, a more promising approach would be to construct and express a single protein consisting of a number of inhibitory domains, using phage display and computer modelling *(58)*.

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Inhibitori proteinaza otvaraju mogućnosti proizvodnje transgenskih krumpira otpornih na bolesti

Sažetak

Inhibitori proteinaza nisu samo važni regulatori endogenih proteaza u biljaka već sudjeluju i u njihovoj obrani od kukaca i patogenih mikroorganizama. Oni su normalno pohranjeni u organima biljke, ali je nakon napada stranih organizama njihova sinteza inducirana u cijeloj biljci. Inhibitori proteinaza inhibiraju vrlo različite probavne proteinaze kukaca, te stoga snizuju brzinu rasta i povećavaju smrtnost ličinki brojnih kukaca. Suvremeni biotehnološki pristup omogućava poboljšanje prirodne obrane uzgajanih biljaka transferom gena koji kodiraju odgovarajući spektar inhibitora proteinaze.