

# The serine protease inhibitors and plant-insect interaction

## *Los inhibidores de serin proteasas y la interacción planta-insecto*

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### ABSTRACT

Plants respond to a physical injury or biological attack by producing, among other compounds, an arsenal of defense proteins, secondary metabolites and phytohormones, all necessary for plant survival. Defense proteins include the group of serine protease inhibitors (SPI), proteins that interact with the active site of their target enzymes. The activity of these protease inhibitors has been exploited to combat insect pests. SPI is an interesting alternative to produce plants with improved resistance characters through selection in the field or expressing their genes in sensitive plants by genetic engineering. The alternative that these natural products offer makes them valuable for the control of several crop pests.

**Key words:** biological control, defense proteins, serine protease inhibitors.

### RESUMEN

*Frente a una agresión física o biológica, las plantas responden bioquímicamente sintetizando, entre otros, un arsenal de proteínas de defensa, metabolitos secundarios y fitohormonas, todos los cuales le permiten sobrevivir. Dentro del grupo de proteínas de defensa se encuentran los inhibidores de serin proteasas (SPI), los cuales interaccionan con el sitio activo de sus enzimas blanco. La actividad de estos inhibidores de proteasas se ha aprovechado en su aplicación para combatir insectos plaga. Los SPI representan una interesante alternativa para producir plantas con mejores características de resistencia, ya sea a través de selección en campo o bien expresando sus genes en plantas sensibles por medio de ingeniería genética. Estos productos innatos de las plantas ofrecen alternativas que los hacen valiosos en el control biológico de plagas de varios cultivos.*

**Palabras clave:** control biológico, inhibidores de serin proteasas, proteínas de defensa.

### Introduction

The attack of pest insects on plants triggers the production of a series of secondary metabolites; defensins, lectins, inhibitors of serine proteases, proteins related to pathogenesis and thionines, among others, all of which constitute the defensive arsenal of the plants (Buchanan *et al.*, 2002). Understanding this defensive arsenal and its mechanism of action will allow us to obtain more alternatives to produce species resistant to pests and reduce the use of synthetic insecticides, which as well as being onerous for the majority of farmers, produce more environmental contamination and induce the appearance of resistant pests (Xavier-Filho, 1992). Genetic engineering currently offers the opportunity to produce plants resistant to insects by the introduction and expression in plants of genes for enteropathogenic proteins (Jouanin *et al.*, 1998).

Among the defense mechanisms of plants are the serine protease inhibitors (SPI), which act on the digestive enzymes trypsin, chymotrypsin, elastase and subtilisin, which have residues of aspartate, serine and histidine in their structure; this gave rise to the name serine proteases. Due to the wide distribution of these enzymes in insect plant pathogens, the SPI represent a mode of protection with a wide range of action (Christeller and Laing, 2005). In 1947, Kunitz began the study of these proteins in soya seeds; in the following years research grew, especially in the area of biological control of pests (Xavier-Filho, 1992).

Given the importance that the applications of the SPI may have in plant biotechnology, the objective of this revision is to summarize the biological functions of the SPI, their mechanisms of action and their effect on pest insects.

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### Ocurrence and distribution of the SPI

The SPI are widely distributed in microorganisms, animals and plants. In plants there are inhibitors for almost all the proteolytic enzymes. The SPI are found in almost all plants; the families Leguminosae and Solanaceae (both dicots) have the largest number of species with these inhibitors. In the monocots the family Graminae has the largest number of species with SPI (Mello, 2001).

The number of SPI is extremely variable among genera, species and varieties. They are concentrated principally in reproductive and reserve organs such as seeds and tubers, although they have also been found in roots, leaves and fruits (Xavier-Filho, 1992). There are currently 9 families of SPI (Table 1), classified according to the homology in their primary sequence, active site, the enzymes on which they

act and their distribution in the vegetable kingdom (Christeller and Laing, 2005).

### Biological applications

In order to feed, insects need enzymes which allow them to acquire the essential amino acids for their growth and development. Lepidopteran larvae need mostly the enzymes similar to the serine proteases (e.g., trypsin, chymotrypsin and elastase), while the coleoptera depend on enzymes similar to cysteine protease (Schuler *et al.*, 1998; Hilder and Boulter, 1999).

The function of the plant protease inhibitors as a defense weapon against insect attack was first demonstrated by Green and Ryan (1972). These authors demonstrated that leaves of potato and tomato were capable of accumulating protease inhibitors

Table 1. Families of serine protease inhibitors (SPI)\*.

Families	Enzymes inhibited	Distribution
1. Kunitz	Trypsin Chymotrypsin Subtilisine Kalikreine Amylase	Leguminosae Gramineae Araceae Alismataceae
2. Bowman-Birk	Trypsin Chymotrypsin Elastase	Leguminosae Gramineae
3. Cucurbitaceae	Trypsin Hageman Factor	Cucurbitaceae
4. Potato I (PPI-1)	Trypsin Chymotrypsin Subtilisine	Solanaceae Gramineae Leguminosae Poligonaceae Cucurbitaceae
5. Potato II (PPI-2)	Trypsin Chymotrypsin	Solanaceae
6. Superfamily of inhibitors of cereals	Trypsin Amylase Hageman Factor	Gramineae Cruciferae Euphorbiaceae Lecitidaceae Leguminosae
7. Mustard trypsin inhibitor	Trypsin	Cruciferae Arabidopsis thaliana
8. Serpin	Trypsin Chymotrypsin Elastase Thrombin	Gramineae, Cucurbitaceae A. thaliana
9. Amylase Inhibitor of $\alpha$ -amylase (ragi) and trypsin of cereal	Trypsin $\alpha$ -amylase	Gramineae

\* Adapted from Shewry and Lucas (1997) and Christeller and Laing (2005).

against the damage provoked by insect attack, as a response to mechanical damage. In a similar study, Gatehouse *et al.* (1979) suggested that the resistance in a crop of caupi (*Vigna unguiculata*) to the attack of the weevil *Callosobruchus maculatus* was due to the high concentration of inhibitors in this cultivar.

Later, the accumulation of protease inhibitors was shown in an experiment that proved that their synthesis is intimately related to a signaling system; a polypeptide called protease inhibitor inductor factor (PIIF) liberated at the injury site (Pearce *et al.*, 1991).

### Mechanism of action

The mechanism of action of the SPI in the digestive tract of insects has not yet been completely elucidated, in large part due to the fact that the direct inhibition of the enzymes does not seem to be their principal effect. The principal factor may be the hypersecretion of the digestive enzymes of the insect caused by their inhibition, which would produce a decrease in essential amino acids (Gatehouse *et al.*, 1993).

All inhibitors have a reactive site which acts on the active site of the target enzyme (Figure 1). For example, the reactive site of the inhibitor of trypsin has arginine and lysine residues, which allow the inhibitor to interact with the enzyme. In this case, the type of inhibition which occurs is competitive inhibition with the substrate (Broadway, 1995). However, there are insects which have changed their digestive enzymes, thus avoiding the action of the

SPI. This occurs in the families Coleopterae and Bruchidae, which do not use serine proteases in the digestion; they use cysteine and aspartic proteases (Gatehouse *et al.*, 1993; Lemos *et al.*, 1990). Pest insects may also have proteolytic enzymes which degrade the inhibitors, and may have mutations which confer greater resistance without losing catalytic activity (Jongsma and Bolter, 1997).

The SPI not only affect digestive enzymes, but also water balance, the development of the insect and its enzymatic regulation (Boulter, 1993). The biological activity of the SPI depends upon (1) the structural compatibility with the protease of the target organism; (2) the physiological conditions in the intestine of the insect (e.g., pH) and (3) the quality and quantity of protein ingested (Broadway, 1995).

Exhaustive studies with the SPI of the Fabaceae, Solanaceae and Poaceae have shown they affect principally Lepidoptera, although they also act on some Coleoptera and Orthoptera. Of the 9 families of SPI, both the inhibitors of Bowman-Birak and those of Kunitz have been administrated successfully in artificial diets, inhibiting the development and growth of larvae of Coleoptera and Lepidoptera (Gatehouse *et al.*, 1993; Johnson *et al.*, 1995).

### Application of spi in genetic engineering

The studies with transgenic plants with SPI genes began when Hilder *et al.* (1987) transformed tobacco plants with the trypsin inhibitor gene (CpTI) of caupi (*Vigna unguiculata*). The results in transgenic tobacco showed mortality and reduced growth in larvae of *Heliothis virescens* (bollworm), as well as lesser damage to the plant. Similar results were obtained against *Heliothis zea* (corn earworm) (Hilder and Boulter, 1999). So far the most active inhibitor has been CpTI, which has been inserted by genetic engineering into at least ten crop plant species. For example, tobacco plants which expressed the CpTI gene caused significant mortality in the corn earworm *Helicoverpa zea*, although its protection was less than that observed with the Bt toxin gene Bt (Schuler *et al.*, 1998). In other tests, tobacco plants transformed with the trypsin inhibitor gene of the bean (*Phaseolus vulgaris*) had leaves capable of decreasing the growth of a number of lepidopteran pests (Hilder *et al.*, 1987).

In the 1990s Gatehouse *et al.* (1993) transformed tobacco plants with the trypsin inhibitor gene of soya (Kunitz family) (SBTI), which showed a

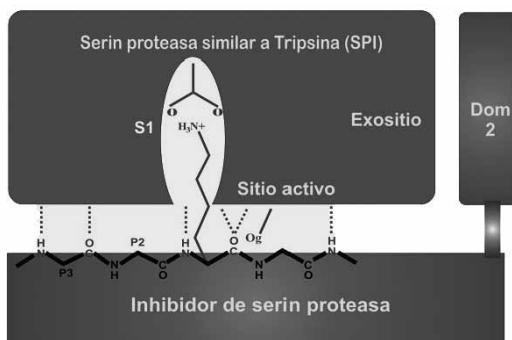


Figure 1. Interaction between the inhibitor and the serine protease similar to the trypsin (TLS) of an insect. The inhibitor has a reactive site with a number of hydrogen bonds that unite with the active sites of the TLS. One lysine residue ( $H_3N^+$ ) in the P1 chain projects into the "bag" S1, in which numerous interactions occur similar to those of the union with the substrate. Taken from Bode and Huber (2000).

high inhibitory effect on the larvae of *H. virescens*. Then McManus *et al.* (1994) transformed tobacco plants with the chymotrypsin inhibitor gene of the potato. These transgenic plants showed resistance to *Chrysodeixis eriosoma* (green measuring worm). In 1996 Duan transformed rice plants with the SPI gene Type 1 of potato, achieving plants resistant to *Sesamia inferens* (pink stem borer) (Ussuf *et al.*, 2001).

In 1997, Yeh *et al.* transformed tobacco plants with the trypsin inhibitor gene of sweet potato. These plants showed a severe effect on the growth of larvae of *Spodoptera litura* (tobacco budworm) (Hilder and Boulter, 1999). In 1999, Lee *et al.* transformed protoplasts of rice with the SBTI gene. The accumulation of this inhibitor was 0.05%-2.5% of the total protein, and the transgenic plants were resistant to *Nilaparvata lugens* (brown planthopper) (Ussuf *et al.*, 2001).

In another study, Altpeter *et al.* (1999) transformed wheat plants with the trypsin inhibitor gene of rye (BTI); these transgenic plants showed resistance to *Sitotroga cerealella* (Angoumoid grain moth). It has been demonstrated that the combination of cysteine and serine protease inhibitors of soya caused inhibition and significant mortality of the larvae of *Tribolium castaneum* (brown flower

beetle.) (Oppert *et al.*, 2005). In 2005, it was shown that the gene SBTI introduced in *Trifolium repens* (white clover) allowed the plant to resist the attacks of *Teleogryllus commodus* (black field cricket) and *Costelytra zealandica* (grass grub) (McManus *et al.*, 2005).

Considering all the evidence which has appeared in the literature (summary in Table 2), the effect that the SPI have on insects is evident; however, negative effects have also been shown on beneficial insects. In a study on the use of SBTI, it was found that they reduced significantly the longevity of bees (*Apis mellifera*), when these feed on inhibitors contained in jellies at concentrations of 1.0%, 0.5% and 0.1% (w/v) (Burgess *et al.*, 1996). It was also demonstrated that 1% SBTI reduced significantly the protein content of the hypofaringeal glands and the activity of the proteolytic enzymes of the intestines of bees (Sagili *et al.*, 2005).

## Conclusions

Beginning with the first study of Kunitz in 1947 with soy seeds, the serine protein inhibitors have become biotechnological tools for the biological control of insect pathogens. Thanks to their great toxicity, countries such as Russia, Japan, USA,

Table 2. Serine protease inhibitors of plant origin transferred by genetic engineering to cultivated plants\*.

Serine protease inhibitors	Plants transformed	Insects on which they act
1. SPI of soya (C-II)	Potato, tobacco, alamo and rape	Coleoptera Lepidoptera
2. Kunitz-type trypsin inhibitor (SKTI)	Potato, tobacco, rice	Lepidoptera and <i>Nilaparvata lugens</i>
3. Rye trypsin inhibitor (CMe)	Tobacco	Lepidoptera
4. Cucurbitaceae trypsin inhibitor (CMTI)	Tobacco	Lepidoptera
5. Cowpea trypsin inhibitor (CpTI)	Apple, lettuce, rape, potato, strawberry, sunflower, yam, tobacco, tomato, rice, cotton, cabbage	Coleoptera Lepidoptera
6. Mustard SPI (MTI-2)	<i>Arabidopsis</i> and tobacco	Lepidoptera
7. Potato SPI Type I (Pot PI-I)	Petunia, tobacco	Lepidoptera Orthoptera
8. Potato SPI Type II (Pot PI-II)	Lettuce, rice, tobacco, birch	Lepidoptera Orthoptera
9. Yam trypsin inhibitor	Tobacco	Lepidoptera
10. Tomato SPI types I and II	Tobacco	Lepidoptera
11. Serpin type SPI	Tobacco	<i>Bemisia tabaci</i>
12. <sup>1</sup> SPI of <i>Brassica oleracea</i> (BoPI)	Tobacco	<i>Heliothis virescens</i>

\* Adapted from Schuler *et al.* (1998); <sup>1</sup>Pulliam *et al.* (2001); Lawrence and Koundal (2002); Fan and Wu (2005).

India, Brazil, etc. are using these defense proteins in their research.

Given that the SPI constitute an option for the control of insect pests, the challenge remains to

perform biochemical studies and studies of biological activity to evaluate those food plants (and/or rescue those in danger of extinction) that may have important SPI activity but are still unknown.

### Literature Cited

- Altpeter, F.; Diaz, I.; Mcauslane, H.; Gaddour, K.; Carbonero, P. and Vasil, I.K.  
1999 Increased insect resistance in transgenic wheat stably expressing trypsin inhibitor CMe. *Molecular Breeding* (5): 53-63.
- Bode, W. y Huber, R.  
2000 Structural basis of the endoproteinase-protein inhibitor interaction. *Biochimica et Biophysica Acta* 1477: 241-252.
- Boulter, D.  
1993 Insect pest control by copying nature using genetically engineered crops. *Biochemistry* (34): 1453-1466.
- Buchanan, B.B., Gruissen, W. y Jones, R.L.  
2002 Biochemistry and Molecular Biology of Plants. *American Soc. of Plant Physiologists*, Rockville, Maryland, USA. 1367p.
- Burgess, E.P.J.; Malone, L.A.; Christeller, J.T.  
1996 Effects of two proteinase inhibitors on the digestive enzymes and survival of honey bees (*Apis mellifera*). *J. Insect Physiol.* 42, 823-828.
- Broadway, R.  
1995 Are insects resistant to plant proteinase inhibitors? *J. Physiol.*, 2: 107-116.
- Christeller, J. y Laing, W.  
2005 Plant serine proteinase inhibitors. *Protein and Peptide Letters* 12:5 pp. 1-9.
- Fan, S-G. y Wu, G-J.  
2005 Characteristics of plant proteinase inhibitors and their applications in combating phytophagous insects. *Botanical Bulletin of Academia Sinica*. 46: 273-292.
- Gatehouse, A.M.R.; Gatehouse, J.A.; Dobie, P.; Kilminster, A.M. y Boulter, D.  
1979 Biochemical basis of insect resistance in *Vigna unguiculata*. *J.Sci. Food Agric.* 30, 948-958.
- Gatehouse, A.; Shi, Y.; Powel, K.; Brough, C.; Hilder, V.; Hamilton, W.; Newell, C.; Merryweather, A.; Boulter, D. y Gatehouse, J.  
1993 Approaches to insect resistance using transgenic plants. *Philosophical transactions of the Royal Society of London Serie B*. 342, pp. 279-286.
- Green, T.R. y Ryan, C.A.  
1972 Wound-induced proteinase inhibitor in plant leaves: a possible mechanism against defense insects. *Science*, 175: 776-777.
- Hilder, V. A.; Gatehouse, A. M. R.; Sheerman, S. E.; Barker, R. F. y Boulter, D.  
1987 A novel mechanism of insect resistance engineered into tobacco. *Nature*, 330: p.169-163.
- Hilder, V.A. y Boulter, D.  
1999 Genetic engineering of crop plants for insect resistance a critical review. *Crop Protection* 18: 177-191.
- Jongsma, M.A. y Bolter, C.  
1997 The adaptation of insects to plant protease inhibitors. *Journal of Insect Physiology* (43): 885-895.
- Johnson, K. A.; Lee, M. J.; Brough, C.; Hilder, V. A.; Gatehouse, A. M. R. y Gatehouse J. A.  
1995 Protease activities in the larval midgut of *Heliothis virescens*: evidence for trypsin an chymotrypsin-like enzymes. *Insect Biochemistry and Molecular Biology.*, 25: 375-383.
- Jouanin, L.; Bonade-Bottino, M.; Girard, C.; Morrot, G. y Giband, M.  
1998 Transgenic plants for insect resistance. *Plant Sci.*, 131:1-11.
- Lawrence, P.K. y Koundal, K.R.  
2002 Plant protease inhibitors in control of phytophagous insects. *Electronic Journal of Biotechnology*. Vol. 5 N° 1: 1-17.
- Lemos, F.J.A.; Campos, F.A.P.; Silva, C.P. y Xavier-Filho, J.  
1990 Proteinase and amylases of larval midgut of *Zabrotes subfasciatus* reared on cowpea (*Vigna unguiculata*) seeds. *Entomol. Exp. Appl.*, 56:219-227.
- McManus, M. T.; White, D. W. R. and McGregor, P. G.  
1994 Accumulation of a chymotrypsin inhibitor in transgenic tobacco can affect the growth of insect pest. *Transgenic Research*, 3, pp. 50-58.
- Mello, G.  
2001 Isolamento e caracterização bioquímica de um inibidor de serinoproteinase de sementes de *Dimorphandra mollis* e o estudo do seu efeito "in vitro" sobre o bruquídeo *Callosobruchus maculatus*. Tese ( Título de mestre em Biologia Funcional e Molecular). Universidade Estadual de Campinas (UNICAMP), Brasil. 94 p.
- Oppert, B.; Morgan, T.D.; Hartzler, K. and Kramer, K.J.  
2005 Compensatory proteolytic responses to dietary proteinase inhibitors in the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Comparative Biochemistry and Physiology Part C* 140, pp. 53-58.
- Pearce, G.; Strydom, D.; Jonhson, S. y Ryan, C.  
1991 A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science*, 253: pp. 895-898.
- Pulliam, D.A.; Williams, D.L.; Broadway, R.M. y Stewart, C.N.  
2001 Isolation and characterization of a serineproteinase inhibitor cDNA from cabbage and its antibiosis in transgenic tobacco plants. *Plant Cell Biotechnology and Molecular Biology*, 2 (1 & 2): 19-32 p.
- Sagili, R.R.; Pankiwand, T. y Zhu-Salzman, K.  
2005 Effects of soybean trypsin inhibitor on hypopharyngeal gland protein content, total midgut protease activity and survival of the honey bee (*Apis mellifera* L.). *Journal of Insect Physiology* 51 (9): 953-957.

Shewry, P.R. y Lucas, J.A.

1997 Plant proteins that confer resistance to pest and pathogens. *Advances in Botanical Research* 26, 135-192.

Schuler, T.H.; Poppy, G.M.; Kerry, B.R. y Denholm, I.

1998. Insect-resistant transgenic plants. *Trends Biotechnol.*, 16: 168-175.

Ussuf, K.K.; Laxmi, N.H. y Mitra, R.

2001 Proteinase inhibitors: plant derived genes of insecticidal protein for developing insect-resistant transgenic plants. *Current Science* 80 (7, 10): 847-853.

Xavier-Filho, J.

1992 The biological roles of serine and cysteine proteinases inhibitors in plants. *Rev. Bras. Fisiol. Vegetal.* 4: 1-6.