Proteinase inhibitors: Plant-derived genes of insecticidal protein for developing insect-resistant transgenic plants

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Proteinase inhibitors (PIs) are anti-metabolic proteins, which interfere with the digestive process of insects. It is one of the important defence strategies existing in plants against predators. The use of the plant-derived *PI* genes for developing insect-resistant transgenic crops has come of age. Several transgenic plants expressing PIs have been created and these plants have shown enhanced resistance against insect pests. Recently PIs have also been used to engineer resistance against viruses in transgenic plants. The current scenario for developing insect, fungal and viral pathogen-resistant transgenic crops by using *PI* gene(s) is reviewed. The biosafety aspect of the transgenic plants expressing PIs is also discussed.

PLANT proteins that inhibit various types of enzymes from a wide range of organisms have been extensively studied[1,2]. Proteinase inhibitors (PIs) comprise one of the most abundant classes of proteins in plants. Most storage organs such as seeds and tubers contain 1 to 10% of their total proteins as PIs, which inhibit different types of enzymes[3,4]. The function of the inhibitors is to control proteolysis within cells, organelles or fluids when limited proteolysis is important for the biochemical or physiological process.

These inhibitors are widely distributed among different botanical families, at least 10 PI families that are specific for each of the four mechanistic classes (Serine, Cysteine, Aspartyl and Metallo) of proteolytic enzymes are known[1,2,5]. The occurrence, classification, physiological significance, effect of PIs on the digestive physiology of animals and the role of PIs as defensive proteins in plants against insects and micro-organisms have been previously addressed in several reviews[1–3,5,6].

One of the recent developments in the field of plant genetic engineering is the manipulation of plants for disease and insect resistance. In an effort to develop insect-resistant crop plants, the role of plant-derived PIs was recognized early, and such resistant transgenic tobacco plants were first reported in 1987 (ref. 7). Since then, genes for inhibitory proteins from different species have been transferred to tobacco, potato, rice, wheat, cauliflower, pea and poplar using the r-DNA techniques. The progress towards developing resistance to insects, fungal and viral pathogens using the genes for proteinase and amylase inhibitors is reviewed.

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Physiological role of PIs

PIs in legume seeds irreversibly inhibit the action of the animal digestive enzymes and hence they are considered antinutritional[2–5]. Besides, there are many important physiological roles for PIs in seeds, which are summarized below.

1. PIs regulate endogenous proteinase levels before and during seed germination for storage protein digestion and to control protein turnover[3,8]. The involvement of PIs in the protection of seed reserves from premature hydrolysis has been established[9,10]. The concentration of inhibitors is reduced during germination, facilitating the hydrolysis of protein for utilization in the germination process.
2. *PI* gene expression has been detected in leaves of several species following wounding, suggesting their role in protecting plants from insect attack and microbial infection. After the identification of PI as a valuable trait suitable for developing insect-resistant transgenic plants, there was intense interest to identify the *PI* gene from different plant species. *PI* gene has been identified and cloned from a wide array of plant sources, including alfalfa[11], tomato[12], potato[13,14], maize[15], mustard[16], poplar[17], tobacco[18], rice[19], sweet potato[20], soybean[21], amaranthus[22], cowpea[23] and barley[24].
3. During seed development, PIs accumulate relatively late, and rapidly increase in the desiccation phase, implying a role in protein stabilization[9,10,25]. Dehydration-related stresses such as drought, salinity and abscisic acid induce the expression of trypsin inhibitors (TIs) in developing seeds of moong bean and lettuce[26–28]. Accumulation of TIs closely resembles that of late embryogenesis abundant protein, which has a specific function in stress dehydration.
4. A new role for PI in the modulation of apoptosis or programmed cell death has been identified in soybean[29].

Cysteine proteinase plays an important role in the regulation of programmed cell death leading to hypersensitive (HR) reaction, following pathogen attack. It has been shown that the ectopic expression of cystatin inhibits the induced cysteine proteinase activity, which in turn blocks programmed cell death[30]. It is suggested that in plants balancing between the cysteine proteinase and cysteine proteinase inhibitor activity regulates the programmed cell death. Thus a new role for PI in modulating the programmed cell death in plants has been identified.

Factors involved in expression of PIs

Plants react to wounding by activating a set of genes, most of them playing a role in wound healing and prevention of subsequent pathogen invasion[31,32]. The gene activation takes place in both wounded and non-wounded plant parts. This systemic accumulation of defence-related products then serves a preventive function, checking the spread of the damaging agent to the healthy part of the plant. The tomato and potato PI families are the best-studied examples of genes, systemically expressed upon wounding.

The detailed analysis of *PI* gene expression in different plant species has shown that it is regulated by a variety of developmental and environmental factors[33–36]. In potato, proteinase inhibitor-II (PI-II) is a multigene family, its constitutive expression in tubers and floral buds and wound-inducible expression in leaves have been reported[35,36]. The mRNA of PI-II appears in stolon of plants induced to tuberize along with other tuber-specific genes such as patatin, and it is present throughout the tuber development. After the dormancy period, no PI-II mRNA was detected in sprouting tubers[36]. This temporal regulation may be responsible for protecting the storage proteins from premature degradation. Studies on the expression of PI from pigeon pea have shown that it is regulated by developmental control[37,38]. A rapid decline in PI belonging to a new species was also observed in horse gram during germination[39].

Environmental factors which influence *PI-II* gene expression have been investigated in detail[33,40–52]. Among the different factors, wound-induced *PI-II* gene activation in potato has received considerable attention. Subsequent research has shown that fungal elicitor, bacterial infection, sucrose, abscisic acid, jasmonic acid and systemin induce *PI-II* gene expression. Factors that have been identified to regulate the expression of *PI-II* gene in potatoes and PI in rice are listed in Table 1.

Analysis of the *PI-II* gene has shown that both developmental expression and wound induction are mediated by specific sequences within the PI-II promoter[53–55]. Deletion analysis from the 3′ to the 5′ end of the promoter region of the wound-inducible *PI-II* gene has identified a 421-base sequence at – 136 to – 557 necessary for expression[53]. Further analysis has identified a 10-base sequence within the 421-base region, which is essential for binding a nuclear protein from wounded potato tissues for *PI-II* gene activation. This 10-base sequence is adjacent to an 8-base consensus sequence at – 147 to – 155 that is present in the promoter region of several other elicitor-inducible genes from various plants[53]. Based on the experimental evidences, it has been suggested that a complex set of *cis* and *trans* acting elements within the – 136 to – 165 region of the *PI-II* gene may be involved in the signalling mechanism that regulate the inducibility of this gene in response to pests and pathogen attack. It was also established by Farmer and Ryan[54] that wound-inducible PI synthesis is regulated by a lipid-based signalling pathway activated by wounding. A model for wound-inducible *PI* gene activation has been proposed in which signal produced by wounding interacts with receptors for activation of signalling cascade[55].

The detailed analysis of wound-inducible promoter of PI-II makes it a potentially useful tool for the genetic engineering of insect and or pathogen resistance in transgenic plants. The advantage of using a systemically inducible promoter to express these well-defined insect and/or pathogen resistance genes in transgenic plants is the high level expression of resistance genes to the period of actual insect or pathogen attack. This will not only conserve the metabolic energy and building blocks of plant cells, but may also minimize the selection pressure on insect pests and pathogen. Thus wound-induced promoter is most desirable for efficient expression of insect resistance genes introduced in transgenic plants.

PIs and alpha amylase inhibitors

Proteins that inhibit α-amylases are found throughout the plant kingdom[1,2]. Many of the abundant proteins in cereal seeds are inhibitors of either α-amylase or PIs, or both[1,2]. The α-amylase inhibitory activities of these proteins are usually directed against α-amylases from animals including a broad spectrum of insects and micro-organisms, but

Table 1. Factors involved in the activation of the *PI* gene in potato and rice

Gene	Factor	Refs
Proteinase inhibitor II (potato)	Wounding	40, 41
	ABA, IAA	42, 43
	Oligourinides	44
	Sucrose	45, 46
	Methyl jasmonate	47
	Jasmonic acid	48
	Fungal elicitor	49
	Bacterial infection	50
	Systemin (peptide)	51
	Electrical potential	52
Cysteine proteinase inhibitor (rice)	Ethylene	98

rarely against plants amylases. A few bifunctional PIs that inhibit both proteinases and α-amylases have been identified[1,2]. These bifunctional inhibitor proteins possess two types of independent reactive sites specific for proteinases as well as for α-amylases and these sites are located on separate regions. These inhibitors tightly bind and inactivate proteinase and α-amylase simultaneously. Thus α-amylase inhibitors from several plants such as maize, rice, ragi, barley, wheat and peas[56–61] are also important component of defence against insects. In a detailed study, α-amylase inhibitors from wheat were able to inhibit amylase isozymes isolated from adults of rice weevil and red flour beetle[62].

Transgenic plants with PI for insect resistance

Transgenic plants expressing foreign insecticidal proteins have been produced for enhanced levels of insect resistance. PIs have been used because of their small size, abundance and stability. They are usually highly specific for a particular class of digestive enzymes of insects. Since the economically important classes of pests like Lepidoptera, Diptera and Coleoptera use serine and cysteine proteinases in their digestive system to degrade proteins in the ingested food, efforts have been focused to use these classes of PIs for developed transgenic plants.

The different PIs that have been used for developing insect-resistant transgenic plants are listed in Table 2. These are cowpea serine PIs (CpTI), potato serine PIs (PI-I and PI-II), sweet potato inhibitors, rice cysteine PIs, soybean Kunitz trypsin inhibitors, corn cystatins, mustard TI, tobacco PI and bean α-amylase inhibitors. Tobacco has been used as model plant in many studies to evaluate the efficacy of PIs as an insecticidal protein, followed by rice, potato, sweet potato, lettuce, tomato, oilseed rape, sunflower, petunia, birch, wheat, cauliflower, poplar pea and azuki bean.

The first *PI* gene to be successfully transferred to tobacco, resulting in enhanced resistance against *Manduca sexta* was *CpTI* gene isolated from cowpea[7]. These studies provided a direct evidence for the effectiveness of *CpTI* against a specific insect. The efficiency of transgenic tobacco plants expressing *CpTI* was also tested against *Spodoptera litura* in feeding trials under laboratory conditions[63]. Reduction to the extent of 50% was observed in the biomass of *S. litura* larvae fed on transgenic leaves expressing 3–5 μg CpTI/g fresh leaves.

Constitutive expression of *CpTI* gene in transgenic rice plants conferred resistance to two species of stem

Table 2. Transgenic plants for enhanced resistance against predators by expression of enzyme inhibitors

Gene source	Transformed plant	Effect evaluated on predators	Refs
Cowpea trypsin inhibitor (*CpTI*)	Tobacco	*Manduca sexta*	7
	Tobacco	*Spodoptera litura*	63
	Rice	*Sesamia inferens, Chilo suppressalis*	64
	Potato	*Lacnobiaoleracea*	99
	Apple	Coleoptera, Lepidoptera	100
	Lettuce, tomato	Coleoptera, Lepidoptera	65
	Oilseed rape	Coleoptera, Lepidoptera	66
	Strawberry	Coleoptera, Lepidoptera	100, 101
	Sunflower	Coleoptera, Lepidoptera	102
	Sweet potato	Coleoptera, Lepidoptera	103
PI-I	Petunia, tobacco	Lepidoptera, Orthoptera	67
Potato *PI-I* and *PI-II*	Tobacco	*Manduca sexta*	68
Potato *PI-II*	Rice	*Sesamia inferens*	70
Potato *PI-II*	Birch, lettuce	Lepidoptera, Orthoptera	65, 66
Potato chymotrypsin inhibitor	Tobacco	*Chrysodeixis eriosoma*	69
Sweet potato *TI*	Tobacco	*S. litura*	20
Sweet potato *TI*	Cauliflower	*Pieris conidia*	71
Rice cysteine inhibitor	Poplar	*Chrysomela tremulae*	72
Soybean Kunitz inhibitor	Rice	*Nilaparvata lugens*	73
Soybean Kunitz inhibitor	Potato, tobacco	Lepidoptera	66, 104
Barley *TI*	Wheat	*Sitotroga cerealella*	74
Corn cystatin	Rice	*Sitophilus zeamatis*	75
Mustard *TI*	*Arabidopsis*, tobacco	Lepidoptera	105
Tobacco *PI*	Tobacco, pea	*Helicoverpa armigera*	76
Cysteine protease inhibitor	Tobacco	Tobacco etch virus, Potato virus	77
Bean α-amylase inhibitor (*Phaseolus vulgaris*)	Pea	*Callosobruchus maculatus*	61
Bean α-amylase inhibitor	Pea	*Bruchus pisorum*	78
Bean α-amylase Inhibitor	Azuki bean	*Callosobruchus chinensis, C. maculatus, C. analis*	79

borers[64]. Expression of *CpTI* gene, driven by the constitutively active promoter of the rice *actin I* gene resulted in high level accumulation of CpTI protein in transgenic rice plants. The TI produced in transgenic plants was biologically active and the plants showed increased resistance against two species of stem borers.

The efficiency of potato PI for insect resistance was evaluated by incorporating the gene into several transgenic plants[65–70]. PI and PI-II expressing transgenic petunia, birch and lettuce plants were resistant to Lepidopteran and Orthopteran insects[65–67]. *PI-I* and *PI-II* genes were transferred to tobacco and the transgenic plants expressing trypsin/chymotrypsin inhibitor were resistant to *M. sexta*[68]. In another study, chymotrypsin inhibitor gene from potato was transferred to tobacco and these plants were also resistant to *Chrysodeixis eriosoma*[69].

PI-II gene from potato was introduced into several Japonica rice varieties and these transgenic plants were found to be insect-resistant in greenhouse trials[70]. Wound inducible PI-II promoter with the first intron of rice *actin I* gene was able to give high level expression of *PI-II* gene in transgenic rice plants. These transgenic plants expressing *PI* gene were found to be resistant to pink stem borer (*Sesamia inferens*). Thus, introduction of plant-derived *PI* gene into cereal plants was successful for control of insect pests.

The sweet potato TI, expressed in transgenic tobacco plants, conferred resistance against *S. litura*[20]. Larval growth of *S. litura*, the tobacco cutworm, was severely retarded compared to its growth on control plants. Transfer of sweet potato *TI*[71] gene to cauliflower made it insect resistant.

cDNA gene encoding a cysteine PI isolated from rice was introduced into tobacco, potato, poplar, oil seed, rape and cotton, but only results reporting the toxicity of such plants against a beetle feeding on poplar have been published[72].

PI gene from different sources have been transferred to rice and the insect resistance has been tested in detail[64,70,73]. Soybean Kunitz trypsin inhibitor was introduced into rice protoplasts[73]. Accumulation level of the protein was found to be 0.05–2.5% of soluble protein; transgenic plants were more resistant to brown plant hopper (*Nilaparvata lugens*).

Recently, insect-resistant wheat was developed by transferring the gene of *barley TI* (*BTI*)[74]; *BTI* accumulation in transgenic wheat was 1.1% of total extracted protein. Significant reduction in the survival of Argoumois grain moth (*Sitotroga cerealella*) was observed. However, only early-instar larvae were inhibited in transgenic seeds and expression of BTI protein in transgenic leaves did not have a significant protective effect against leaf-feeding insects.

Corn cystatin (CC) cDNA under the control of AMV 35 S promoter in rice plants showed a high level of expression in both rice seeds and leaves. About 2% of the total proteins in transgenic rice plants were found to be CC proteins. It showed strong inhibitory activity against insect gut proteases of *Sitiophilus zeamais*[75] and a wide inhibitory spectrum against various cysteine proteinases of insects.

A cDNA clone of multidomain PI from *Nicotiana alata* was transferred into tobacco and peas under the control of promoter from ribulose 1,5-biphosphate carboxylase small subunit gene[76]. *Helecoverpa armigera* larvae that ingested transgenic tobacco or pea leaves showed higher mortality or delayed growth and development relative to the control larvae.

A recent report[77] that rice cysteine proteinase inhibitor oryzacystatin engineered into tobacco provided resistance against potyvirus marks another important development. The processing of all known potyviruses involves the activity of cysteine proteinases. The inhibition of constitutive expression of cysteine proteinase by cystatin could induce resistance against two important potyviruses, tobacco etch virus (TEV) and potato virus Y (PVY). There was a good correlation between the level of oryzacystatin protein and resistance to TEV and PVY at all levels tested. The inhibitor was ineffective against tobacco mosiac virus as processing of this virus does not involve cysteine proteinases. These results suggest that the plant cystatins can be used against different potyviruses and potentially also against other viruses, whose replication involves cysteine proteinase activity.

Corn TI was also found to be effective against a group of plant pathogenic fungi[88]. In a detailed study, the corn TI was over-expressed in *E. coli* to obtain large quantities of the active recombinant inhibitor, to test the efficiency against various plant pathogenic fungi. The recombinant corn TI inhibited both conidium germination and hyphal growth of all nine plant pathogenic fungi studied, including *Aspergillus flavus*, *A. parasiticus* and *Fusarium moniliforme*. These studies suggest that corn TI could be useful for developing fungal disease resistance.

It has been shown that transgenic pea expressing bean α-amylase inhibitor from bean (*Phaseolus vulgaris*) confers resistance to the bruchid beetle, *Callosobruchus maculatus* and *C. chinensis*[61]. Bean α-amylase inhibitor expressing pea was also resistant to *Bruchus pisorum*[79]. In another study, the expression of bean α-amylase inhibitor in azuki bean conferred resistance to three species of bruchids[80]. However the bifunctional α-amylase/proteinase inhibitor genes are more useful for developing insect-resistant transgenic plants.

In the studies mentioned above, the efficiency of the expression of introduced *PI* gene was found to be sufficient to impart resistance to the challenged insects. In a majority of the cases, cauliflower mosaic virus 35 S RNA promoter was used for expressing *PI* gene. Promoters from ribulose 1,5-biphosphate carboxylase small subunit gene, rice actin gene, wound-inducible *PI-II* gene and

ubiquitin gene were also found to be equally or more efficient for producing the protein in transgenic plants.

These studies have conclusively demonstrated the feasibility of genetic engineering of insect resistance by expressing *PI* genes. Recent field trials carried out in the US showed that the expression of *CpTI* in tobacco provided significant protection in the field against *Helicoverpa zae*[81]. The field trials were conducted in parallel with transgenic plants expressing *Bacillus thuringiensis* (*Bt*) toxin, *cry IAC* gene. The result from these studies has shown that *CpTI* gene has a negative effect on both larvae survival and plant damage. *CpTI*-transgene showed variability from one trial to another, suggesting that the degree of protection provided by the *CpTI* gene in transgenic plants can be affected by factors such as plant age, environmental condition and heterogeneity of insect population. In another field experiment, tobacco expressing the serine-proteinase inhibitor from soybean resulted in up to 100% mortality of first-instar cotton-leaf worms (*Spodoptera littoralis*) (M. Delledonne *et al.*, unpublished). However, resistance levels achieved by the same constructs in potato were much lower, resulting mainly in retarded growth of *S. littoralis* rather than mortality. Although a number of transgenic crop plants have been developed to determine the usefulness of different PIs, the detailed analysis of these transgenic plants expressing different PIs is not available or not published due to patent applications[82]. To date, no crop expressing PI transgenes has been commercialized.

PIs are highly specific for a particular class of digestive enzymes. However, insects have shown enough flexibility to switch proteinases composition in their guts to overcome the particular proteinase inhibitor expressed in the transgenic plants[83]. Evidences have been presented to show that insects can adapt to the ingestion of PIs[84]. Insects belonging to both Lepidoptera and Coleoptera can overexpress existing gut proteinases or induce the production of new types that are insensitive to the introduced PIs to overcome the deleterious effect of PI ingestion[85]. This may be contributing for the decreased effectiveness of the PIs expressed in transgenic plants. In a recent study it was shown that high level expression of soybean trypsin inhibitor gene in transgenic tobacco plants failed to confer resistance against *H. armigera*[86]. It is known that gut digestive enzymes are not the only targets affected by PIs; they can also affect the water balance, moulting and enzyme regulation of insects[87].

Biosafety of transgenic plants expressing PIs

The biosafety and risk assessment of the environmental release of genetically modified plants have been addressed in detail in several recent reviews[88–91]. Limited information on the biosafety evaluation of genetically modified food incorporating PI is available. Since these PIs are from plant-derived genes and are easily inactivated by cooking, introduction of the *PI* gene into new host crops can be regarded as a safe strategy from the food safety standpoint. Another important factor is that the gene transfer to other species will not create any new environmental hazard.

The nutritional value of transgenic peas expressing bean amylase inhibitor has been investigated in detail[92]. It had minimal detrimental effect on the nutritional value of peas fed to rats at 30% of the diet. This study indicated that such transgenic peas could be used in a diet at 300 g/kg level without harmful effects on growth.

Future directions

The technology for developing insect-resistant transgenic plants is expanding very rapidly. Such plants have the potential to become a part of the integrated pest management systems in future. With the development of several transgenic plants expressing *Bt* toxin it emerged that codon usage of *Bt* is far from optimal for expression in plants. Making partially or totally synthetic genes suitable for plant expression circumvents this problem[93]. Plant-derived genes such as *PIs* will have the advantage of efficient expression in transgenic plants. Transgenic crops expressing PIs are going to play an important role in future in insect pest management strategies. It is shown that *Bt* can promote the evolution of resistance if allowed to exert consistent selection pressure on the target insects[94]. A suggested solution for preventing/delaying development of resistance against *Bt* is to utilize genes encoding PIs in combination with *Bt*. This possibility has been examined in several systems[95–97] and the results are encouraging. The resistance against introduced PIs from transgenic plants can develop in insects by increasing the secretion of proteolytic enzyme or by inducing new types of proteases. Development of new types of PIs by protein engineering with altered affinity for different proteases, and incorporating multidomain PIs in a single gene are attractive strategies for developing insect-resistant transgenic plants in future.

1. Garcia-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, L., Royo, J. and Carbonero, P., *Oxford Surv. Plant Mol. Cell Biol.*, 1987, **4**, 275–334.
2. Ryan, C. A., *Annu. Rev. Phytopathol.*, 1990, **28**, 425–449.
3. Ryan, C. A., in *The Biochemistry of Plants* (ed. Marcus, A.), Academic Press, New York, 1981, vol. 6, pp. 351–370.
4. Pearce, G., Sy, L., Russsel, C., Ryan, C. A. and Hass, M., *Arch. Biochem. Biophys.*, 1982, **213**, 456–462.
5. Ryan, C. A., *BioEssays*, 1990, **10**, 20–23.
6. Jouanin, L., Bonade-Bottino, M., Girard, C., Morrot, G. and Giband, M., *Plant Sci.*, 1998, **131**, 1–11.
7. Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F. and Boulter, D., *Nature*, 1987, **300**, 160–163.
8. Baumgartner, B. and Chrispeels, M. J., *Plant Physiol.*, 1976, **58**, 1–6.

9. Kirsi, M., *Physiol. Plant.*, 1974, **32**, 89–93.
10. Collins, J. L. and Sanders, G. G., *J. Food Sci.*, 1976, **41**, 168–172.
11. McGurl, B., Mukherjee, S., Kahn, M. and Ryan, C. A., *Plant Mol. Biol.*, 1995, **27**, 995–1001.
12. Lee, J. S. *et al.*, *Proc. Natl. Acad. Sci. USA*, 1986, **83**, 7277–7281.
13. Cleveland, T. E., Thornburg, R. W. and Ryan, C. A., *Plant Mol. Biol.*, 1987, **8**, 199–207.
14. Sanchez-Serrano, J. J., Schmidt, R., Schell, J. and Wilmitzer, L., *Mol. Gen. Genet.*, 1986, **203**, 15–20.
15. Rohrmeier, T., Lehle, L. and WIJP, I., *Plant Mol. Biol.*, 1993, **22**, 783–792.
16. Ceci, L. R., Spoto, N., de Virgilio, M. and Gallerani, R., *FEBS Lett.*, 1995, **364**, 179–181.
17. Hollick, J. B. and Gordon, M. P., *Plant Mol. Biol.*, 1993, **22**, 561–572.
18. Atkinson, A. H., Heath, R. L., Simpson, R. J., Clarke, A. E. and Anderson, M. A., *Plant Cell*, 1993, **5**, 203–213.
19. Abe, K., Emori, Y., Kondo, H., Suzuki, K. and Arai, S., *J. Biol. Chem.*, 1988, **262**, 16793–16797.
20. Yeh, K. W., Lin, M. I., Tuan, S. J., Chen, Y. M., Lin, C. Y. and Kao, S. S., *Plant Cell Rep.*, 1997, **16**, 696–699.
21. Song, S. I., Kim, C. H., Back, S. J. and Choi, Y. D., *Mol. Cells*, 1991, **1**, 317–324.
22. Rodriguez, E. V., Labra, A. B., Benicio, G. G., Boradenenko, A., Estrella, A. H. and Simpson, J., *Plant Mol. Biol.*, 1999, **41**, 15–23.
23. Lee, J. S. *et al.*, *Proc. Natl. Acad. Sci. USA*, 1986, **83**, 7277–7281.
24. Odani, S., Koide, T. and Ono, T., *J. Biol. Chem.*, 1983, **258**, 7998–8003.
25. Domosey, C., Welham, T., Sidebottom, C. and Firmin, J. L., *FEBS Lett.*, 1995, **360**, 15–20.
26. Lam, J. M., Pwe, K. H., Sun, W. Q., Chua, Y. L. and Wang, X. J., *Plant Sci.*, 1999, **142**, 209–218.
27. Downing, W. L., Mauxion, F., Fauvrque, M. O., Reviron, M. P., de Vienne, D., Vartanian, N. and Giraudat, J., *Plant J.*, 1992, **2**, 685–693.
28. Xu, D., Duan, X., Wang, B., Hong, B., Ho, D. T. and Wu, R., *Plant Physiol.*, 1996, **110**, 249–257.
29. Kosslak, R. M., Chamberlin, M. A., Palmer, R. G. and Bowen, B. A., *Plant J.*, 1997, **11**, 729–745.
30. Solomon, M., Belenghi, B., Delledonne, M., Menachem, E. and Levine, A., *Plant Cell*, 1999, **11**, 431–443.
31. Bowles, D. J., *Annu. Rev. Biochem.*, 1990, **59**, 873–907.
32. Lamb, C. J., *Plant Cell Environ.*, 1982, **5**, 471–475.
33. Keil, M., Sanchez-Serrano, J. J. and Willmitzer, L., *EMBO J.*, 1989, **8**, 1323–1330.
34. Pena-Cortes, H., Wilmitzer, C. and Sanchez-Serrano, J. J., *Plant Cell*, 1991, **3**, 963–972.
35. Pena-Corte, H., Sanchez-Serrano, J. J., Rocha-Sosa, M. and Willmitzer, L., *Planta*, 1988, **174**, 84–89.
36. Sanchez-Serrano, J. J. *et al.*, in *Biotechnology in Plant Disease Control*, 1993, pp. 157–173.
37. Godbole, S. A., Krishna, T. G. and Bhatia, C. R., *J. Sci. Food Agric.*, 1994, **64**, 87–92.
38. Godbole, S. A., Krishna, T. G. and Bhatia, C. R., *J. Sci. Food Agric.*, 1994, **66**, 497–503.
39. Sreerama, Y. N., Das, J. R., Gowda, L. R. and Rao, D. R., in Proceedings of the International Meeting on Molecular Mechanism of Toxicity, Osmania University, Hyderabad, 1997, pp. 56–63.
40. Green, T. R. and Ryan, C. A., *Science*, 1972, **175**, 776–777.
41. Bradshaw, H. D., Hollick, J. B., Parsons, T. J., Clarke, H. R. G. and Gordon, M. P., *Plant Mol. Biol.*, 1989, **14**, 51–59.
42. Pena-Cortes, H., Sanchez-Serrano, J. J., Mertens, R., Willmitzer, L. and Prat, S., *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 9851–9855.
43. Kernan, A. and Thronburg, R. W., *Plant Physiol.*, 1989, **91**, 73–78.
44. Bishop, P. D., Makus, D. J., Pearce, G. and Ryan, C. A., *Proc. Natl. Acad. Sci. USA*, 1981, **78**, 3536–3540.
45. Johnson, R. and Ryan, C. A., *Plant Mol. Biol.*, 1990, **14**, 527–536.
46. Kim, S. R., Kim, Y., Costa, M. A. and An, G., *Plant Phiosiol.*, 1992, **98**, 1479–1483.
47. Farmer, E. E., Johnson, R. R. and Ryan, C. A., *Plant Physiol.*, 1992, **98**, 995–1002.
48. Farmer, E. E. and Ryan, C. A., *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 7713–7716.
49. Rickauer, M., Fournier, J. and Esquerre-Tougage, M. T., *Plant Physiol.*, 1989, **90**, 1065–1070.
50. Pautot, H., Holzer, F. M. and Walling, L. L., *Mol. Plant–Microb. Int.*, 1991, **4**, 284–292.
51. Pearce, G., Strydom, D., Johnson, S. and Ryan, C. A., *Science*, 1991, **253**, 895–898.
52. Stankovic, B. and Davies, E., *FEBS Lett.*, 1996, **390**, 275–279.
53. Palm, C. J., Costa, M. A., An, G. and Ryan, C. A., *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 603–607.
54. Farmer, E. E. and Ryan, C. A., *Plant Cell*, 1992, **4**, 129–134.
55. Ryan, C. A., *Plant Mol. Biol.*, 1992, **19**, 123–133.
56. Richardson, M., Valdes-Rodriguez, S. and Blanco-Labra, A., *Nature*, 1987, **327**, 432–434.
57. Tashiro, M. and Maki, Z., *J. Nutr. Sci. Vitenol.*, 1979, **25**, 255–264.
58. Shivraj, B. and Pattabiraman, T. N., *Indian J. Biochem. Biophys.*, 1980, **17**, 181–185.
59. Sanchez-Monge, R., Gomez, L., Garcia-Olmedo, F. and Salcedo, G., *FEBS. Lett.*, 1986, **207**, 105–109.
60. Maeda, K., *Biochem. Biophys. Acta*, 1986, **871**, 250–256.
61. Shade, R. E., Schroder, H. E., Pueyo, J. J., Tabe, L. M., Murdock, L. L., Higgins, T. J. V. and Chrispeels, M. J., *Biotechology*, 1994, **12**, 793–796.
62. Chen, M. S. *et al.*, *Insect Biochem. Mol. Biol.*, 1992, **22**, 261–268.
63. Sane, V. A., Nath, P., Aminuddin and Sane, P. V., *Curr. Sci.*, 1997, **72**, 741–747.
64. Xu, D., Xue, Q., McElory, D., Mawal, Y., Hilder, V. A. and Wu, R., *Mol. Breed.*, 1996, **2**, 167–173.
65. Gatehouse, A. M. R., in *Global Plant Genetic Resources for Insect Resistant Crops* (eds Clement, S. L. and Quisenberry, S. S.), CRC Press, 1999.
66. Burgess, E. P. J. and Gatehouse, A. M. R., in *Biotechnology and the Improvement of Forage Legume* (eds McKersie, B. D. and Brown, D. C. W.), CAB International, pp. 229–258.
67. Beuning, *et al.*, Annual Queenstown Molecular Biology Meeting, 1991.
68. Johnson, R., Narvaez, J., An, G. and Ryan, C. A., *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 9871–9875.
69. McManus, M. T., White, D. W. R. and McGregor, P. G., *Transgen. Res.*, 1994, **3**, 50–58.
70. Duan, X., Li, X., Xue, Q., Abo-El-Saad, M., Xu, D. and Wu, R., *Nat. Biotechnol.*, 1996, **14**, 494–498.
71. Ding, L. C., Hu, C. Y., Yeh, K. W. and Wang, P. J., *Plant Cell Rep.*, 1998, **17**, 854–860.
72. Leplé, J. C. *et al.*, *Mol. Breed.*, 1995, **1**, 319–328.
73. Lee, S. I. *et al.*, *Mol. Breed.*, 1999, **5**, 1–9.
74. Altpeter, F., Diaz, I., McAuslane, H., Gaddour, K., Carbonero, P. and Vasil, I. K., *Mol. Breed.*, 1999, **5**, 53–63.
75. Irie, K., Hosoyama, H., Takeuchi, T., Iwabuchi, H., Watanabe, H., Abe, M. and Arai, S., *Plant Mol. Biol.*, 1989, **30**, 149–157.
76. Charity, J. A., Anderson, M. A., Bittisnich, D. J., Whitecross, M. and Higgins, T. J. V., *Mol. Breed.*, 1999, **5**, 357–365.
77. Campos, R. G., Acosta, J. A. T., Arias, L. J. S. and Lim, M. A. G., *Nat. Biotechnol.*, 1999, **17**, 1223–1226.
78. Chen, Z. Y., Brown, R. L., Lax, A. R., Cleveland, T. E. and Russin, J. S., *Appl. Environ. Microbiol.*, 1999, **3**, 1323–1324.
79. Shroeder, H. E. *et al.*, *Plant Physiol.*, 1995, **107**, 1233–123.
80. Ishimoto, M., Sato, T., Chrispeels, M. J. and Kitamura, K., *Entomol. Exp. Appl.*, 1996, **79**, 309–315.
81. Hoffmann, M. P. *et al.*, *J. Econ. Entomol.*, 1992, **85**, 2516–2522.

82. Schuler, T. J., Poppy, G. M., Kerry, B. R. and Denholm, L., *TIBTECH*, 1998, **16**, 168–175.
83. Jongsma, M. A., Bakker, R. L., Peters, J., Bosch, D. and Stiekema, W. J., *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 8041–8045.
84. Jongsma, M. A. and Bolter, C., *J. Insect Physiol.*, 1997, **43**, 885–895.
85. Gatehouse, A. M. R., Butler, K. J., Fenton, K. A. and Gatehouse, J. A., *Entomol. Exp. Appl.*, 1985, **39**, 279–286.
86. Nandi, A. K., Basu, D., Das, S. and Sen, S. K., *Bioscience*, 1999, **24**, 445–452.
87. Boulter, D., *Phytochemistry*, 1993, **34**, 1446–1453.
88. Bhatia, C. R. and Mitra, R., *PINSA, B64*, 1998, **5–6**, 293–318.
89. Ghosh, P. K. and Ramanaiah, T. V., *J. Sci. Ind. Res.*, 2000, **59**, 114–120.
90. Ammann, K., Jacot, Y. and Almazyad, P. R., Asia Pacefic Workshop on Biosafety: Environmental Impact Analysis of Transgenic Plants, M.S. Swaminathan Research Foundation, Chennai, 21–26 July 1997.
91. Ferrarolo, G., Report, biosafe@icgeb.triesta.it.http
92. Pusztai, A., Barodocz, G. G., Alonso, R., Chrispeels, M. J., Schroeder, H. E., Tabe, L. M. and Higgins, T. J., *J. Nutr.*, 1999, **129**, 1597–1603.
93. Mazier, M., Pannetier, C., Tourneur, J., Jouanin, L. and Giband, M., *Biotechnol., Annu. Rev.*, 1997, **3**, 313–347.
94. McGaughey, W. H., *Agric. Econ. Environ.*, 1994, **49**, 95–102.
95. Santos, M. O., Adang, M. J., All, J. N., Boerma, H. R. and Parrott, W. A., *Mol. Breed.*, 1997, **3**, 183–194.
96. MacIntosh, S. C., Stone, T. B., Jokerst, R. S. and Fuchs, R. L., *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 8930–8933.
97. Cormu, D. *et al.*, in *Somatic Cell Genetics and Molecular Genetics of Trees* (eds Boerjan, W. and Neale, D. B.), Kluwer Academic Publishers, Dordrecht, 1996, p. 131–136.
98. Cervantez, E., Rodriguez, A. and Colas, G. N., *Plant Mol. Biol.*, 1994, **25**, 207–214.
99. Gatehouse, A. M. R. *et al.*, *Mol. Breed.*, 1997, **3**, 1–15.
100. James, D. J. *et al.*, *Phytoparasitica*, 1992, **20**, 835–878.
101. Graham, J., McNicol, R. J. and Greig, K., *Ann. Appl. Biol.*, 1995, **127**, 163–173.
102. USDA/APHS Biotechnology Permit Database (http://aphis.usda.gov./bbep/bp/).
103. Newell, C. *et al.*, *Plant Sci.*, 1995, **107**, 215–227.
104. Marchetti, S. *et al.*, *Potato Res.*, 1994, **37**, 450–451.
105. Jouanin, L., pers. commun.

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