

Short communication

Potato cysteine proteinase inhibitor gene family: molecular cloning, characterisation and immunocytochemical localisation studies

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Abstract

Potato cysteine proteinase inhibitors (PCPIs) represent a distinct group of proteins as they show no homology to any other known cysteine proteinase inhibitor superfamilies, but they all belong to the Kunitz-type soybean trypsin inhibitor family. cDNA clones for five PCPIs have been isolated and sequenced. Amino acid substitutions occurring in the limited regions forming loops on the surface of these proteins suggest a further classification of PCPIs into three subgroups. Accumulation of PCPI was observed in vacuoles of stems after treatment with jasmonic acid (JA) using immunocytochemical localisation, implying that these inhibitors are part of a potato defence mechanism against insects and pathogens. Genomic DNA analysis show that PCPIs form a multigene family and suggest that their genes do not possess any introns.

Abbreviations: CPI, cysteine proteinase inhibitor; JA, jasmonic acid; NID, novel inhibitor of cathepsin D; PCPI, potato cysteine proteinase inhibitor; PDI, potato cathepsin D inhibitor; PI-I, potato inhibitor I; PI-II, potato inhibitor II; PKPI, potato Kunitz-type proteinase inhibitor; STI, soybean Kunitz trypsin inhibitor

The family of cysteine proteinase inhibitors from potato (PCPIs) represents a distinct group of proteins as they show no homology to either of the known cysteine proteinase inhibitor superfamilies, the cystatin superfamily [35] and the recently characterised thyroglobulin type-1 domain-like inhibitors [3]. They also do not possess any primary structure elements so far known to be responsible for the inhibition of cysteine proteinases. Their primary structure shows them to be related to the Kunitz-type soybean trypsin inhibitor

(STI) family [19]. Although they are potent inhibitors of lysosomal cysteine proteinases, some of them also possess weak inhibitory activity against serine proteinases trypsin and chymotrypsin [6]. Inhibition of proteinases belonging to two different classes is a very rare feature among proteinase inhibitors. The physiological role of proteinase inhibitors in plants has been the subject of intense research over several years. A model for their role as part of the plant defence mechanism that is activated in response to insect attack or other severe mechanical wounding was postulated for serine proteinases inhibitors of the potato inhibitors I (PI-I) and II (PI-II) families [9, 10]. Jasmonic acid (JA) has been shown to be a messenger molecule in the sig-

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession numbers U59272, U59273, U59274, U59276 and U59277 for PCPI10, PCPI8, PCPI7, PCPI5 and PCPI3, respectively.

nal transduction pathway of plant defence response to wounding [9]. Insect larvae feeding on a diet including inhibitors are retarded in their development and sometimes they are unable to reach their reproductive phase [4].

The aim of this study was to further characterise PCPIs at the cDNA level, to elucidate their gene organisation, and to assess their biological function. Immunocytochemical localisation studies were performed to establish the principle of sorting and accumulation of PCPIs.

Isolation and characterisation of PCPI cDNA clones

Two primers (5'-CATGAATTCATATGCTTGTACTCCCTGAAGTT-3' and 5'-CCGGATCCTACGCCTTGTAACAC-3') were constructed which correspond to the 5' and 3' ends of the mature protein-coding region of p34021 [30], a cDNA clone with the deduced sequence identical to the best characterised potato cysteine proteinase inhibitor, PCPI 8.3 [19]. The potato tuber (cv. Ulster Sceptre) λ gt11 cDNA library [33] was screened using a probe obtained by PCR which was 94% identical to the mature protein-coding region of p34021 cDNA [30]. Five cDNA clones isolated that way were sequenced and further characterised (PCPI3, PCPI5, PCPI7, PCPI8 and PCPI10). None of them has a complete 5' end, although that does not interfere in the structural analysis of their coding region. They share between 94.2% and 99.7% of identical nucleotides (insertions and deletions excluded). The deletions in the coding region do not result in frameshifts. Three AATAAA polyadenylation signals are present in the 3'-untranslated region (UTR) of all isolated cDNA clones, of which the second and third overlap. The occurrence of overlapping polyadenylation signals has already been observed in some plant genes [22]. Protein and cDNA sequences homologous to PCPIs were obtained through database search [1] and are listed in Figure 1. Ishikawa *et al.* [16] has proposed a classification of potato Kunitz-type proteinase inhibitors (PKPI) into PKPI-A, PKPI-B and PKPI-C homology groups. Serine proteinase inhibitors and serine/aspartic proteinase inhibitors from potato are members of both PKPI-A and PKPI-B groups. PCPIs belong to PKPI-C group which therefore includes serine, serine/cysteine and cysteine proteinase inhibitors. We propose a further classification of these proteins into three subgroups on the bases of characteristic sequence regions specific for subgroup and of percentage of amino acid identity (Figure 1). Nucleotide deletions that are typ-

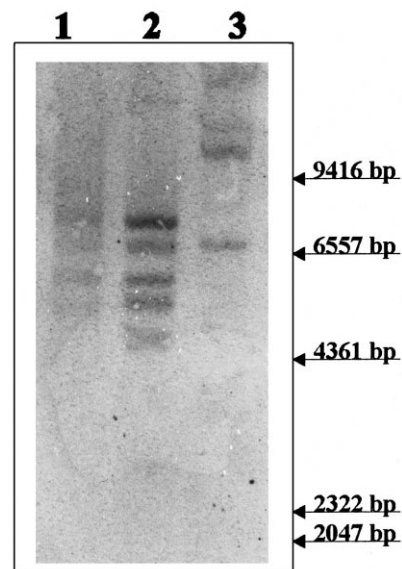


Figure 3. Southern blot analysis of potato (cv. Ulster Sceptre) genomic DNA for the PCPI gene family. Genomic DNA was digested with *Eco*RI (lane 1), *Eco*RI/*Bam*HI (lane 2) and with *Bam*HI restriction endonucleases (lane 3) and hybridised with labelled PCPI8 cDNA.

ical for subgroup II cDNAs only, also support this classification. The percentage of amino acid identity within subgroup I members is above 93%, and above 86% within subgroup II. PCPI 6.6 [6] exhibits only 39.5% of identity to members of subgroup I or II and was assigned to the third subgroup on the basis of its cysteine proteinase inhibitory activity. The comparison of nucleotide and deduced amino acid sequences of PCPIs shows a very low percentage of silent mutations (11.7%). Thus, one can assume that the positive selection has been in favour of diversification of this group of proteins.

The position of Cys residues in proteins coded by the newly isolated cDNA is generally preserved as is found for other members of the STI family. Some post translational modifications occur on PCPI proteins [16, this study]. No potential glycosylation sites were found in any of the PKPI-C members, thus the PKPI-A group remains the only one of the PKPIs so far known with a potential N-glycosylation site. Interestingly, potential phosphorylation and sulphatation sites were found in proteins of PCPI subgroup I and II (PC-GENE, IntelliGenetics USA) that are not found in other PKPI proteins.

STI family members interact with their target enzymes at one or two reactive sites [29]. The reactive

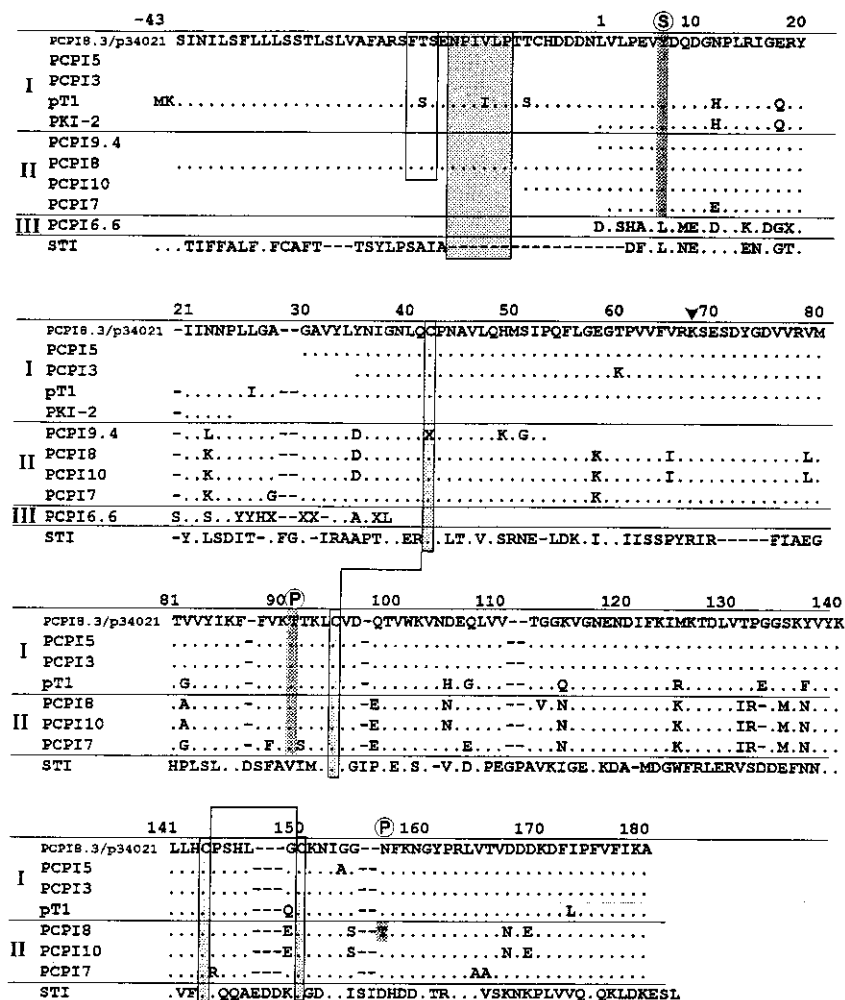


Figure 1. Amino acid sequence alignment of PKPI-C proteins and STI [17]. Subgroup I includes p34021 [30] (PCPI 8.3 [19] is identical to the mature protein coding region of this clone), PCPI5*, PCPI3*, pT1 [37], PKI-2 [36]; Subgroup II includes PCPI 9.4 [6], PCPI8*, PCPI10*, PCPI7* and subgroup III includes PCPI 6.6 [6], (* this study). The 22 kDa group of proteins characterised by Suh *et al.* [31] have the same N-terminal sequence as PCPI 8.3. The numbering used is of PCPI 8.3. Points indicate amino acid residues identical to those of p34021 and PCPI 8.3. Dashes indicate amino acid deletions. The vacuolar sorting signal is indicated by the shaded box. The potential recognition site for signal peptide cleavage is marked by the open box. Connected boxes represent potential disulphide bridges. P denotes a potential phosphorylation site and S a potential sulphatation site. ∇ indicates the reactive site for trypsin inhibition in STI.

site for serine proteinase inhibition was proven to be Arg at position 63 (STI numbering) [17]. The second reactive site for proteinase inhibition was predicted to be located in the C-terminal domain in the loop within the second disulphide bridge [32]. It is interesting that subgroup I and subgroup II members differ in the putative cysteine proteinase inhibitory site at Pro-145 → Arg or Gly-148 → Glu. Since several different activities of STI members have been identified (cathepsin D inhibitors [23, 28], storage protein sporamin A [13], double-headed wheat α -amylase/subtilisin inhib-

itor [21], drought stress-induced proteins [12, 20], dehydroascorbate reductase [34]), it is possible that PKPIs have some other function beside inhibitory activity, which has not yet been observed. Because of its structural diversity, the C-terminal domain is more likely to be the candidate for additional functions. One of the most interesting regions is that between Ile-124 and Tyr-139. According to the three-dimensional structure of STI this region is predicted to form a loop and is significantly different in PKPI-C I and II.

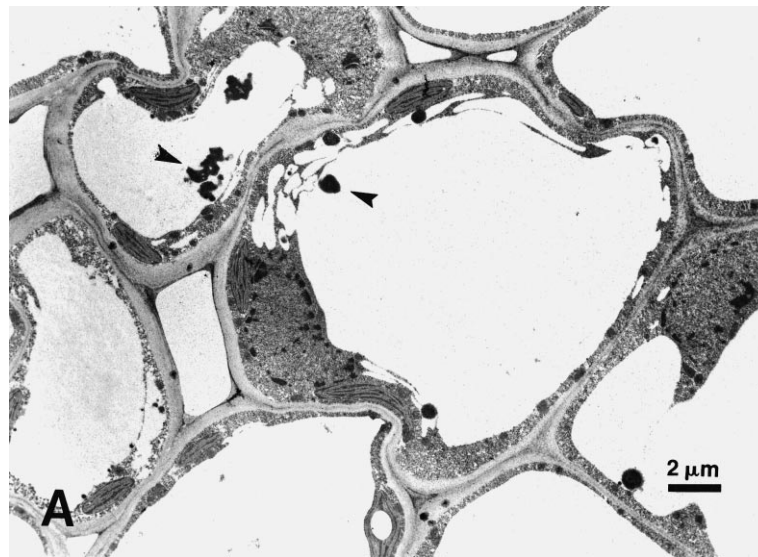


Figure 2. Immunocytochemical localisation of PCPI 6.6 in stem tissue. **A.** A low magnification view of stem cells (plants grown on the medium supplemented with 100 μ m JA). **B.** Immunogold labelling of PCPI 6.6 associated with membraneless protein aggregates in the vacuolar space of stem. **C.** Localisation in the intercellular space of stem. **D.** A section treated with the non-immune serum was used as control. V, vacuole; P, protein aggregates; CW, cell wall; IS, intercellular space; C, cytoplasm; Bar = 200 nm (unless indicated otherwise). Purified rabbit antibodies to PCPI 6.6 ($c=0.5$ mg/ml) [6] were tested for cross-reactivity with NID by western blot analysis (results not shown). Arrowheads are pointing towards some individual gold particles or some groups of gold particles associated with PCPI 6.6.

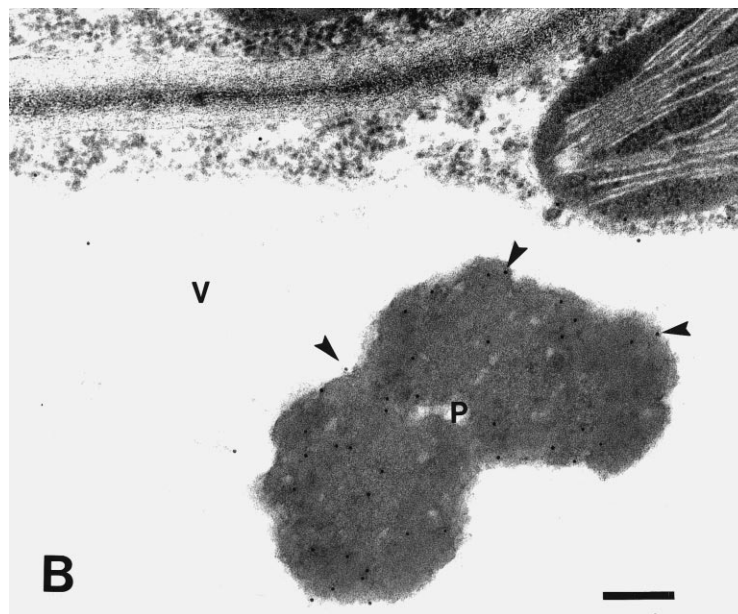


Figure 2. Continued.

Intracellular localisation of PCPIs

The preproregion of PCPIs was analysed by PC-GENE and by amino acid comparison to other STI proteins

(Figure 1). The N-terminal end of the preproregion (prepeptide) consists of a highly hydrophobic cluster that is predicted to be a transmembrane spanning segment (Ile-3 to Ala-20, p34021 numbering). This is

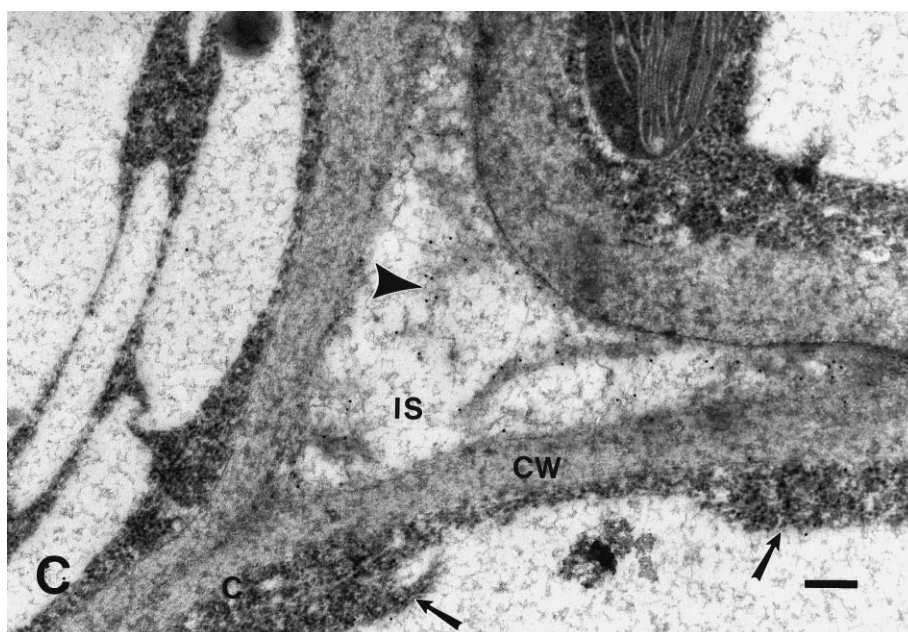


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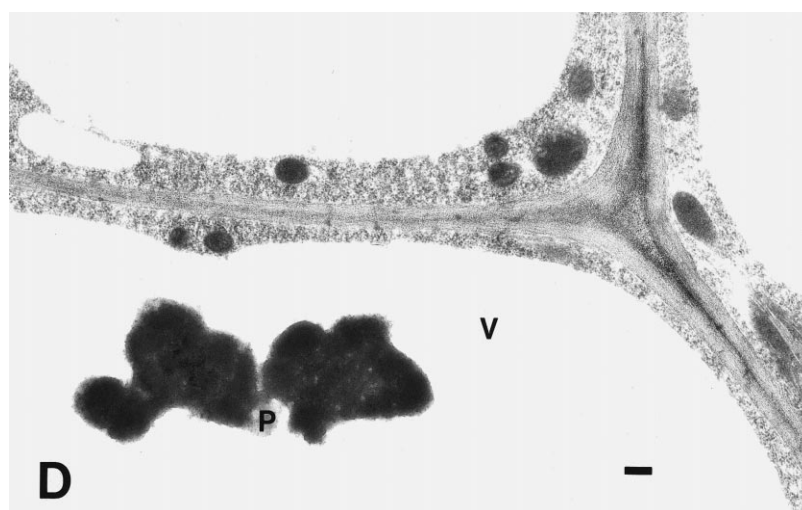


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thought to be an endoplasmic signal sequence [16] with a potential signal peptide cleavage site Phe-Thr/Ser-Ser [16, 24]. The pro part of the PCPI prepropeptide contains a conserved Asn-Pro-Ile-Xxx-Leu-Pro motif which represents the vacuolar sorting signal [7, 25].

Gold immunolabelling of thin sections of stem tissues was performed to show the intracellular localisation of PCPI 6.6 (Figure 2) basically following the procedure described [27]. Gold particles were associated with membraneless protein bodies of amorphous

character within the cell vacuole (Figure 2B). A smaller amount of gold-labelling was also found in the cytoplasm (Figure 2C). Additionally, specific immunogold labelling of PCPI 6.6 was observed in the intercellular space (Figure 2C). Narvez-Vasques *et al.* have shown that the PI-I and PI-II family inhibitors can be deposited in the cell wall matrix of secretory and epidermal cells of the root cap besides their accumulation in the vacuole [26]. However, there is only one report of their presence in the intercellular space. It was demonstrated

by gold immunolabelling that NID is accumulated in the intercellular space of potato root cells of a plant grown in non-inducible conditions (medium without JA) [18] in addition to accumulation of the protein in the vacuole of JA treated plants [27]. At the same time, NID was detected associated with the Golgi apparatus which may transport it out of the cell where it could represent the first defence against a potential penetrating pathogen [18].

Genomic DNA analysis

Genomic DNA of two different potato cultivars, *Solanum tuberosum* L. cv. Ulster Sceptre and cv. Désirée was isolated from leaves of a single specimen following the procedure described [11]. Southern blot analysis of potato cysteine proteinase inhibitor genes was performed with the digoxigenin-labelled PCPI8 cDNA [this study] using the genomic DNA of cv. Ulster Sceptre. The results shown in Figure 3 suggest that a moderate size multigene family of potato cysteine proteinase inhibitors exists. The emergence of multigene families can be explained as the evolutionary answer to the need of the plant to produce large amounts of proteins in a short time span [14].

PCR amplification of cv. Désirée genomic DNA was performed using the same primers as for preparation of the probe for screening. It yielded a product of about 550 bp. The size matches the size of PCPI cDNA amplified by the same primers (results not shown), therefore the data suggest that most or all PCPI genes are intronless at least in the mature protein coding region. The same was shown also for other PKPI proteins [2, 16]. It has been suggested that intronless genes may have evolved in order to be able to respond without significant delay to various exogenous signals [8].

Expression of PCPI genes

The levels of PKPI proteins have been shown to be developmentally and environmentally regulated [5, 16, 32]. mRNA of p34021 and pT1 accumulates during early tuber development [32, 37]. In agreement with this results, in our study, an unusually high percentage of positive clones (2%) was obtained on screening a tuber cDNA library. It has also been confirmed that p34021 mRNA accumulates in leaves upon wounding [32]. In addition to this, strong immunolabelling of PCPI 6.6 was observed in vacuoles of the leaf tissues grown on media supplemented with 100 μ M JA (Figure 2A and 2B) compared to control medium (res-

ults not shown). The induction by JA was observed in different families of proteinase inhibitors, such as PI-I, PI-II [9], plant cystatins [15] and members of the PKPI-A group of the STI family [16, 33]. Since PCPI proteins are also induced by JA, that would imply that they also belong to the set of proteins responding in the same way to exogenous signals. Genomic DNA studies, in addition, suggest that expression of PCPI genes is regulated by exogenous signals in order to produce large quantities of proteins without significant time delay.

The mechanism of induction of PCPI genes, together with their role in different stress conditions in plants remains to be clarified. Another unsolved problem is the mechanism of inhibition of cysteine proteinases by PCPIs as is their ability to inhibit both serine and cysteine proteinases. Any potential functions of potato cysteine proteinase inhibitors beside their inhibitory activity also remain to be discovered.

Acknowledgements

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References

1. Altschul SF, Gish W, Miller Q, Myers EW, Lipman DJ: Basic local alignment search tool. *J Mol Biol* 215: 403–410 (1990).
2. Barlič-Maganja D, Štrukelj B, Pungerčar J, Gubenšek F, Turk V, Kregar I: Isolation and sequence analysis of the genomic DNA fragment encoding an aspartic proteinase inhibitor from potato (*Solanum tuberosum* L.). *Plant Mol Biol* 20: 311–313 (1992).
3. Bevec T, Stoka V, Pungerčič G, Dolenc I, Turk V: MHC class II-associated p41 invariant fragment is a strong inhibitor of lysosomal cathepsin L. *J Exp Med* 183: 1331–1338 (1996).
4. Broadway RM, Duffey SS: The effect of plant protein quality on insect digestive physiology and the toxicity of plant proteinase inhibitor. *J Insect Physiol* 34: 1111–1117 (1988).
5. Brzin J, Kidrič M: Proteinases and their inhibitors in plants: role in normal growth and in response to various stress conditions. *Biotechnol Genet Engng Rev* 12: 421–467 (1995).
6. Brzin J, Popovič T, Drobnič-Košorok M, Kotnik M, Turk V: Inhibitors of cysteine proteinases from potato. *Biol Chem Hoppe-Seyler* 369: 233–238 (1988).
7. Chrispeels MJ, Raikhel NV: Short peptide domains target proteins to plant vacuoles. *Cell* 68: 613–616 (1992).
8. D'Onofrio M, Lee DM, Starr CM, Miller M, Hanover JH: The gene encoding rat nuclear pore glycoprotein p62 is intronless. *J Biol Chem* 266: 11980–11985 (1991).

9. Farmer EE, Ryan CA: Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* 4: 129–134 (1992).
10. Farmer EE, Caldelari D, Pearce G, Walker-Simmons MK, Ryan CA: Diethylthiocarbamic acid inhibits the octadecanoid signalling pathway for the wound induction of proteinase inhibitors in tomato leaves. *Plant Physiol* 106: 337–342 (1994).
11. Gelvin SB, Schilperoort RA, Verma DPS: *Plant Molecular Biology Manual*. Kluwer Academic Publishers, Dordrecht (1989).
12. Gosti F, Bertauche N, Vartanian N, Giraudat J: Absciscic acid-dependent and -independent regulation of gene expression by progressive drought in *Arabidopsis thaliana*. *Mol Gen Genet* 246: 10–18 (1995).
13. Hattori T, Nakagawa S, Maeshima m, Nakamura K, Asahi T: Molecular cloning and nucleotide sequence of cDNA for sporamin, the major soluble protein of sweet potato tuberous roots. *Plant Mol Biol* 5: 313–320 (1985).
14. Heidecker GT, Messing J: Structural analysis of plant genes. *Annu Rev Plant Physiol* 37: 439–466 (1986).
15. Hildmann T, Ebner M, Pena-Cortes H, Sanchez-Serano J, Willmitzer L, Prat S: General roles of abscisic and jasmonic acid in gene activation as a result of mechanical wounding. *Plant Cell* 4: 1157–1170 (1992).
16. Ishikawa A, Ohta S, Matsuoka K, Hattori T, Nakamura K: A family of potato genes that encode Kunitz-type proteinase inhibitors: structural comparison and differential expression. *Plant Cell Physiol* 35: 303–312 (1994).
17. Kim S-H, Hara S, Hase S, Ikenaka T, Toda H, Kitamura K, Kaizuma N: Comparative study of amino acid sequences of Kunitz-type soybean trypsin inhibitors, Ti^a , Ti^b , and Ti^{c1} . *J Biochem* 98: 435–448 (1985).
18. Kregar I, Ravnikar M, Pungerčar J, Poljšak-Prijatelj M, Štrukelj B: Proteinase inhibitors are part of plant defence mechanism. *Proceedings of IBA, Rogla* pp. 103–112 (1994).
19. Križaj I, Drobnič-Košorok M, Brzin J, Jerala R, Turk V: The primary structure of inhibitor of cysteine proteinases from potato. *FEBS Lett* 333: 15–20 (1993).
20. Lopez F, Vansuyt G, Fourcroy P, Casse-Delbart F: Accumulation of a 22-kDa protein and its mRNA in the leaves of *Raphanus sativus* in response to salt stress or water deficit. *Physiol Plant* 91: 605–614 (1994).
21. Maeda K: The complete amino acid sequence of the endogenous α -amylase inhibitor in wheat. *Biochim Biophys Acta* 871: 250–256 (1986).
22. Manen JF, Simon P: A possible explanation for the multiple polyadenylation sites in transcripts coding for a winged-bean leghemoglobin. *Planta* 191: 289–292 (1993).
23. Mareš M, Meloun B, Pavlik M, Kostka V, Baudyš M: Primary structure of cathepsin D inhibitor from potatoes and its structure relationship to soybean trypsin inhibitor family. *FEBS Lett* 251: 94–98 (1989).
24. Matsouka K, Matsumoto S, Hattori T, Machida Y, Nakamura K: Vacuolar targeting and post-translational processing of the precursor to the sweet potato tuberous root storage protein in heterologous plant cells. *J Biol Chem* 265: 19750–19757 (1990).
25. Nakamura K, Matsouka K: Protein targeting to the vacuole in plant cells. *Plant Physiol* 101: 1–5 (1993).
26. Narvaez-Vasquez J, Franceschi VR, Ryan CA: Proteinase-inhibitor synthesis in tomato plants: Evidence for extracellular deposition in roots through secretory pathway. *Planta* 189: 257–266 (1993).
27. Ravnikar M, Štrukelj B, Poljšak-Prijatelj M, Mavrič I, Meško P, Pungerčar J, Kregar I: Immunolocalisation of aspartic proteinase inhibitors in potato shoots induced by jasmonic acid *in vitro*. *Acta Pharm* 45: 139–145 (1995).
28. Ritonja A, Križaj I, Meško P, Kopitar M, Lučovnik P, Štrukelj B, Pungerčar J, Buttle DJ, Barrett AJ, Turk V: The amino acid sequence of a novel inhibitor of cathepsin D from potato. *FEBS Lett* 267: 13–15 (1990).
29. Shibata H, Hara S, Ikenaka T: Purification and characterisation of proteinase inhibitors from winged bean (*Psophocarpus tetragonolobus* (L.) DC.) seeds. *J Biochem* 99: 1147–1155 (1986).
30. Stiekema WJ, Heidekamp F, Dirkse WG, Beckum JV, Haan PD, Bosch CT, Louwerse JD: Molecular cloning and analysis of four potato tuber mRNAs. *Plant Mol Biol* 11: 255–269 (1988).
31. Suh S-G, Peterson JE, Stiekema WJ, Hannapel DJ: Purification and characterisation of 22-kilodalton potato tuber proteins. *Plant Physiol* 94: 40–45 (1990).
32. Suh S-G, Stiekema WJ, Hannapel DJ: Proteinase-inhibitor activity and wound-inducible gene expression of the 22-kDa potato-tuber proteins. *Planta* 184: 423–430 (1991).
33. Štrukelj B, Pungerčar J, Meško P, Barlič-Maganja D, Gubenšek F, Kregar I, Turk V: Characterisation of aspartic proteinase inhibitors from potato at the gene cDNA and protein levels. *Biol Chem Hoppe-Seyler* 373: 477–482 (1992).
34. Trumper S, Follmann H, Haberlein I: A novel dehydroascorbate reductase from spinach chloroplasts homologues to plant trypsin inhibitor. *FEBS Lett* 352: 159–162 (1994).
35. Turk V, Bode W: The cystatins: protein inhibitors of cysteine proteinases. *FEBS Lett* 285: 213–219 (1991).
36. Walsh TA, Twitchell WA: Two Kunitz-type proteinase inhibitors from potato tubers. *Plant Physiol* 97: 15–18 (1991).
37. Yamagishi K, Mitsomuri C, Kikuta Y: Nucleotide sequence of a cDNA encoding the putative trypsin inhibitor in potato tuber. *Plant Mol Biol* 17: 287–288 (1991).