Pseudothionin-St1, a potato peptide active against potato pathogens

Manuel MORENO, Ana SEGURA and Francisco GARCÍA-OLMEDO
Laboratorio de Bioquímica y Biología Molecular, ETS Ingenieros Agrónomos, Madrid, Spain

A 5-kDa polypeptide, pseudothionin *Solanum tuberosum* 1(Pth-St1), which was active against *Clavibacter michiganensis* subspecies *sepedonicus*, a bacterial pathogen of potatoes, has been purified from the buffer-insoluble fraction of potato tubers by salt extraction and HPCL. Pth-St1 was also active against other potato pathogens tested (*Pseudomonas solanacearum* and *Fusarium solani*). The N-terminal amino acid sequence of this peptide was identical (except for a N/H substitution at position 2) to that deduced from a previously reported cDNA sequence (EMBL accession number X-13180), which had been misclassified as a Bowman-Birk protease inhibitor. Pth-St1 did not inhibit either trypsin or insect α-amylase activities, and, in contrast with true thionins, did not affect cell-free protein synthesis or β-glucuronidase activity. Northern-blot and tissue-print analyses showed that steady-state mRNA levels were highest in flowers (especially in petals), followed by tubers (especially in the epidermal cell layers and in leaf primordia), stems and leaves. Infection of leaves with a bacterial pathogen suspended in 10 mM $MgCl_2$ switched off the gene, whereas mock inoculation with 10 mM $MgCl_2$ alone induced higher mRNA levels.

Plant storage tissues are protected against pathogens and predators by high constitutive levels of toxic or inhibitory proteins, including chitinases, glucanases, protease and α-amylase inhibitors, ribosome-inactivating proteins, thionins and others [1–4]. Such proteins are often induced or increased well above basal levels in other parts of the plant, such as leaves, in response to attack by pathogens or insects (see [1]). In the course of a systematic screening of antipathogenic proteins from tubers, we have isolated a cysteine-rich polypeptide that is active against potato pathogens and belongs to a group of homologous proteins [5–14] for which different *in vitro* activities have been observed, such as inhibition of protein synthesis [5, 6], of α-amylases [7] or of plant pathogens [13, 14]. We also report on the expression of the corresponding gene during normal development and in response to infection.

MATERIALS AND METHODS

Biological materials

Solanum tuberosum cv. Desireé was cultivated at 60% humidity and 20°C, with a photoperiod of 18 h light/6 h dark. Bacterial pathogens *Clavibacter michiganensis* subsp. *sepedonicus*, strain C5, and *Pseudomonas solanacearum*, strain P2, as well as the fungal pathogen *Fusarium solani*, strain 1, were from the ETSIA collection. *Pseudomonas syringae* DC 3000 pv. tomato was the kind gift of Dr J. Ryals (Ciba-Geigy Corp.). An extract from larvae of the storage pest *Tenebrio molitor* was the gift of our colleague G. García-Casado. Insects *Blatta orientalis* (adult cockroaches) and *Locusta migratoria* (mature larvae) were the gift of Dr C. Gutierrez (CSIC).

Purification and characterization of the protein

Frozen tuber material (20 g) was ground to powder in liquid nitrogen, using a mortar and pestle, and extracted once with 80 ml buffer (0.1 M Tris/HCl, 10 mM EDTA, pH 7.5) and twice with 80 ml H_2O. The resulting pellet was then extracted with 50 ml 1.5 M LiCl at 4°C for 1 h, and the extract dialyzed against 5 l H_2O, using a Spectra/Por 6 (MWCO:3000) membrane, and freeze-dried. The extract was subjected to reverse-phase HPLC (RP-HPH) as previously described [15]. The proteins were subjected to SDS/PAGE in preformed gradient gels (4–20%; BioRad) according to the manufacturers' instructions. Amino acid sequencing was carried out by automated Edman degradation of the intact proteins.

Protein synthesis and enzyme-inhibition assays

Preparations of rabbit reticulocyte lysate (Promega) were incubated with 1 µl 19 unlabeled amino acids, 25 µCi [^{35}S]methionine and 1 µg tobacco mosaic virus (TMV) RNA. Incubations were for 60 min at 30°C. Aliquots of 2 µl were spotted at different times on Whatmann 3 MM paper, air dried and treated with 10% trichloroacetic acid. Filters were washed twice with 10% trichloroacetic acid, once with ethanol, once with ethyl ether and dried. Radioactivity was measured in a liquid-scintillation spectrometer.

Inhibition tests of β-glucuronidase were performed by Dr M. Piñeiro as previously described [16]. Crude α-amylase extracts from the insects *Tenebrio molitor*, *Blatta orientalis* and *Locusta migratoria*, and from human saliva were prepared and assayed as previously described, but on a smaller

scale [17]. Wheat monomeric and dimeric α-amylase inhibitors were the gift of Drs R. Sanchez-Monge and G. Salcedo (Madrid, Spain). Trypsin from bovine pancreas (Serva), Kunitz (Serva) and Bowman-Birk (Sigma) soybean trypsin inhibitors were used. Trypsin assays were performed as described by Boisen and Djurtoft [18], but with a tenfold reduction of the scale.

Pathogen inhibition tests and plant inoculations

Inhibition tests *in vitro* were carried out at the protein concentrations indicated in Fig. 3 and as previously described [15]. The thionin used as positive control in inhibition experiments was a mixture of α-thionins and β-thionins from wheat endosperm (gift of Dr A. Molina, Madrid, Spain). Plant inoculations were performed as follows. *P. syringae* DC 3000 was grown overnight in N-1 medium (0.5% peptone, 0.1% beef extract, 0.2% yeast extract, 0.5% NaCl, pH 7.2), diluted in 10 mM $MgCl_2$ to 10^8 colony-forming units/ml, and inoculated at multiple points in the leaves with a blunt-ended syringe (20 µl/point). Mock inoculation with 10 mM $MgCl_2$ was carried out in the same manner. Leaves were collected at given times and frozen in liquid nitrogen.

RNA extraction, Northern-blot and tissue-print hybridization.

A DNA probe encoding Pth-St1 was prepared by the PCR amplification. Complementary DNA was generated by reverse transcription of 1 µg potato tuber mRNA and used as a PCR template following standard procedures [20]. Two oligonucleotides were designed, based on the published cDNA sequence [8], with added *Eco*RI sites to simplify subsequent fragment cloning (number 4100, 5′-GAATTCTTAGCATGGCTTAGTGC-3′; number 4101, 5′-GAATTCATGCGTTTCTTTGC-3′). Amplification conditions were 94 °C, 1 min; 45 °C, 1 min; 72 °C, 30 s; 30 cycles. The amplified fragment was cloned in pUC19, sequenced and random labelled with ^{32}P-ATP following standard procedures [20].

RNAs were purified from frozen tissues by phenol/chloroform extraction, followed by precipitation with 3 M LiCl [20]. Electrophoresis was carried out on 5% formaldehyde/agarose gels, which were blotted to Hybond N membranes (Amersham). Dot blots were performed according to current protocols [20]. Hybridization and washing were carried out at 65 °C according to Church and Gilbert [21]. Equal sample loads were checked by hybridizing with a ribosomal cDNA probe, excising the dots and determination of the radioactivity. Radioactive signals in Northern-blot experiments were quantitated by densitometry for three independent filters.

Tissue-print hybridization was carried out essentialy as described by McClure and Guilfoyle [22] and Varner et al.[23]. Two Hybond-N membranes were printed on both sides of a fresh, thin organ section and used for hybridization, while a Hybond-C membrane was printed on the adjacent free side of the organ and used for amido-black staining. Membranes were air dried and cross-linked by treatment with UV light prior to hybridization. As a negative control, a wheat cDNA probe of similar size was used. This probe encoded the 3′ end (257 bp) of the SS2 sucrose synthase cDNA and was the gift of Dr P. Sánchez de la Hoz (Madrid, Spain). Hybridization conditions were the same as in RNA blot experiments.

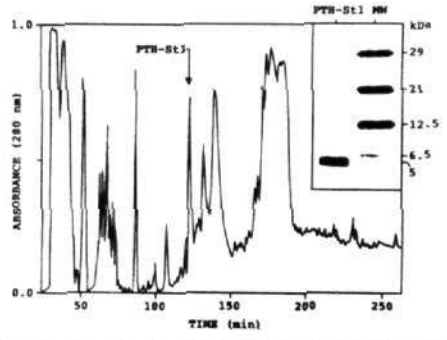

Fig. 1. Fractionation by RP-HPLC of salt-extracted proteins from crude cell wall preparations from potato tubers. The peak corresponding to Pth-St1 is indicated. Inset, SDS/PAGE of Pth-St1. Apparent molecular masses (kDa) of marker proteins are indicated on the right.

```
PTH-St1   RNCESLSHRFKGPCTRDSNC
P322      RHCESLSHRFKGPCTRDSNCASVCET.KHFSGGN..CHG.FRRR.GPCTKPC
SE60      RVCESQSHGFHGLCNRDSNCALVCRN.EGFSGGR..CKRS..RR.GPCTRIC
SIA2      RVCMGKSAGFKGLCMRDQNCAQVCLQ.EGWGGGN..CDGVMRQ..CKGIRQCW
G1H       RICRRRSAGFKGPCVSNKNCAQVCMQ.EGWGGGN..CDGPLRR..CKGMRRC
G2P       KVCRQRSAGFKGPCVSDKNCAQVCLQ.PGWGGGN..CDGPFRR..CKGIRQC
G1P       KICRRRSAGFKGPCWSNQNCAQVCQQ.EGWGGGN..CDGPFRR..CKDIRQD
SIA3      RVGRRRSAGFKGLCMSDHNCAQVCLQ.EGWGGGN..CDGVIRQ..CKGIRQCW
FST       RECKTESNTFPGICITKPPCRKACIS.KKFTDGH..CSKLLKR..CLGIKPC
pI230     NTCENLAGSYKGVCPGG..GDRHCRTQEGAISGR..CRDDFR...CWGIKNC
pSAS10    KTCENLVDTYRGPCFTTGSGDDHCKNKEHLLSGR..CRDDVR...CWGIRNC
SIA1      RVCMGKSQHHSFPCISSRLCSNEGVK.EKGGWTAGRCELRY....CRQKAC
pI39      NTCEHLADTYRGVCFTNASCDDHCKNKAHLISGT..CHDWK....CPGTQNC
```

Fig. 2. Alignment of the N-terminal amino acid sequence of Pth-St1 with that deduced from the nucleotide sequence of the p322 cDNA [8], as well as with the following sequences: SE60 from soybean [11]; SIA2,3,1 from sorghum [7]; G1H, G2P, G1P from barley and wheat [5, 6]; FST from tobacco [10]; pI230, pI139 from pea [9]; pSAS10 from cowpea [12]. The sequences are listed in order of increasing divergence with respect to Pth-St1.

RESULTS

Purification and characterization of PTh-St1

A crude cell wall preparation was obtained from potato tubers by pelleting a low-salt, neutral-buffer homogenate and washing it twice with H_2O. This preparation was extracted with 1.5 M LiCl and the extract fractionated by RP-HPLC, as shown in Fig. 1. The protein fractions were screened for their ability to inhibit *in vitro* growth of the pathogen *C. michiganensis* subsp. *sepedonicus* (at 100 µg/ml) and subjected to SDS/PAGE. An electrophoretically homogeneous active protein was identified (inset in Fig. 1) and its N-terminal amino acid sequence was determined up to the 20th residue. The sequence did not present heterogeneity and was identical to that deduced from the nucleotide sequence of a previously cloned cDNA (EMBL accession number X-13180), except for a substitution (N/H) at position 2, probably due to the different cultivars used in each case. As shown in Fig. 2, the amino acid sequence of Pth-St1 was clearly homologous to those of other proteins previously reported in other plant species, either directly determined or deduced from nucleotide sequences of the corresponding cDNAs. The yield of this protein, designated PTh-St1, was about 1.5 mg/kg fresh mass.

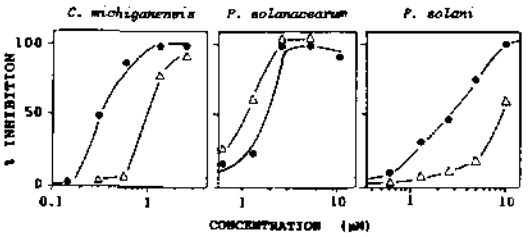

Fig. 3. Inhibition curves of Pth-St1 (●) and thionin (△) against the indicated pathogens.

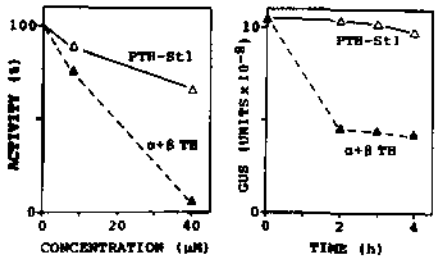

Fig. 4. Inhibition of cell-free protein synthesis (left) and of β-glucuronidase GUS (right) by Pth-St1 (△) and by thionin (▲).

Inhibitory properties of Pth-St1

Growth-inhibition curves for the purified Pth-St1 against two bacterial and one fungal potato pathogen are shown in Fig. 3. The protein was more active than the thionin control against *C. michiganensis* and *F. solani*, whereas it was about equally active against *P. solanacearum*.

The protein was not inhibitory towards trypsin, even at concentrations up to 30-fold higher than those required for inhibition by Bowman-Birk or Kunitz trypsin inhibitors from soybeans, or towards α-amylases from insect species and human saliva, at concentrations up to 20-fold higher than those that were effective for the cereal inhibitors used as controls (not shown). In contrast with thionins, Pth-St1 was only slightly inhibitory of cell-free protein synthesis and had no effect on β-glucuronidase activity (Fig. 4).

Expression of the gene encoding Pth-St1

A DNA probe encoding Pth-St1 was obtained by PCR amplification, using primers that were based on the previously published cDNA sequence [8], followed by cloning in pUC19. The sequence of the cloned DNA (237 bp) corresponded to that of the previously reported cDNA. RNAs were extracted from different parts of the plant, subjected to Northern-blot analysis (Fig. 5A), and quantified by dot-blotting serial dilutions of equal amounts of these RNAs (Fig. 5B). Expression within tissues and organs was further investigated by hybridization of the same DNA probe to tissue prints of appropriate sections (Fig. 6). The gene was expressed not only in tubers and stems, as previously reported [8], but also at higher levels in flowers, specially in petals, and at a lower level in leaves. Expression in tubers was highest in leaf primordia and in the periphery, while in stems a strong signal was associated with the vascular inner face.

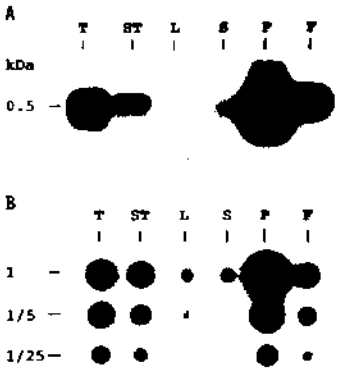

Fig. 5. Expression of the gene encoding Pth-St1. (A) Northern blot, 5 μg total RNA in each slot. (B) Dot blot, 1 = 2 μg of total RNA. T, tuber; ST, stem; L, leaf; S, sepal; P, petal; F, flower bud.

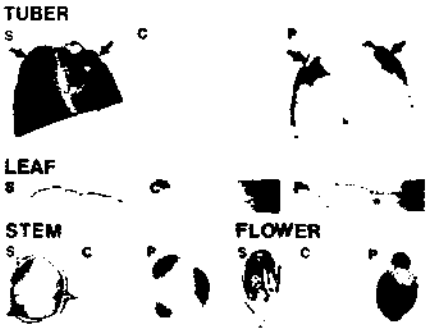

Fig. 6. Tissue-print analysis of the distribution of Pth-St1 mRNA in the indicated tissues. Sections stained with amido black (s), hybridized with control probe (c) and with Pth-St1 probe (p). Arrows indicate positions of flower primordia.

Heavy inoculation of potato leaves with a strain of *P. syringae* causing hypersensitive reaction led to a marked decrease in steady-state mRNA levels in the infected leaves and of neighbouring uninfected leaves (with a certain lag

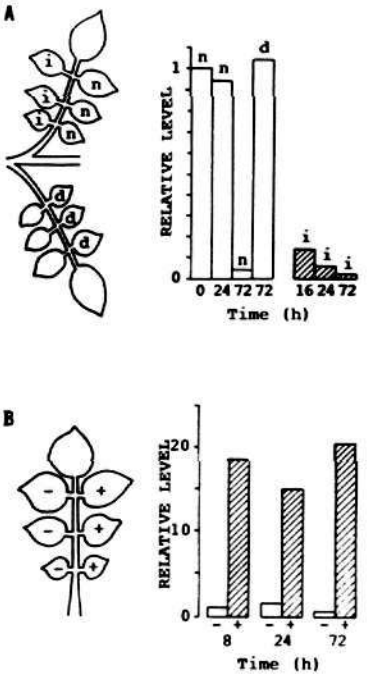

Fig. 7. Steady-state Pth-St1 mRNA levels in response to infection by *P. syringae* DC3000 and to mock inoculation. (A) Infected leaves (i); neighbouring non-infected leaves (n); distal non-infected leaves (d). (B) Mock inoculation with 10 mM $MgCl_2$ (+); not inoculated (−).

time), but did not affect more distant leaves of the same plant (Fig. 7A). In contrast, mock inoculation with 10 mM $MgCl_2$, the medium used to suspend the bacterial inoculum, led to a 20-fold mRNA increase (Fig. 7B).

DISCUSSION

The N-terminal amino acid sequence of Pth-St1 was essentially identical to that deduced for the longest open-reading frame of clone p322 (EMBL accession number X-13180), which had been misclassified as a Bowman-Birk protease inhibitor [8]. The sequence has none of the highly-conserved features of Bowman-Birk inhibitors [2], while it is clearly homologous to a family of proteins, of which the first one to be reported was designated γ-thionin [5, 6]. This was an inadequate name because α-thionin and β-thionin are two closely related variants of just one of the five types of thionins described [4], and the so-called γ-thionin was not homologous to any of them, although it is also basic, cysteine rich and of similar size. The separation from Bowman-Birk inhibitors and from true thionins is further justified because Pth-St1 did not inhibit trypsin, even at much higher concentrations than those required for true Bowman-Birk inhibitors, and had little or no effect on cell-free protein synthesis or on β-glucuronidase, at concentrations that are inhibitory in the case of true thionins.

It has been reported that some homologues of Pth-St1 are inhibitory towards insect α-amylases [7], although no actual data has been published. No such activity was detected for Pth-St1 either against the enzymes from three different insect species or against the human salivary enzyme, which suggests that inhibition of α-amylases is not a general property of the family and that previous reports of such a property for other members of the family should be re-examined.

The *in vitro* activity of Pth-St1 against pathogens that affect potato, such as the three tested in this work, further indicated a possible defense role for this protein and was in line with the antipathogenic properties reported for Pth-St1 homologues from the Cruciferae [13, 14]. Chelation of Ca^{2+} has been proposed as the inhibition mechanism for this family [13]; the fact that Pth-St1 is more active than the control thionin against two of the pathogens and about equally active against another suggests that the two protein families do not share the same mechanism of action, as otherwise their relative activities should be determined by their relative capacities to bind the cation. It also suggests that the two types of proteins could complement each other in protecting the plant against pathogens.

Expression patterns of the gene encoding Pth-St1 in the course of normal development in tubers, stems, leaves and flowers are congruent with a defense role. Infection with a strain of *P. syringae* that caused hypersensitive reaction not only switched off the basal level of expression but also prevented its induction above basal levels by 10 mM $MgCl_2$. This probably represents a mechanism by which at least some pathogens can attempt to overcome the constitutive defense barriers of the plant and for which a few examples have been reported in the literature [24, 25]. The fact that, in this case, the mechanism is concomitant with an incompatible interaction indicates that its occurrence is not sufficient for compatibility and is independent of the activation of positive defense mechanisms and of the hypersensitive reaction.

Amino acid sequences determined at Ciba Geigy Corp., advice given by Drs R. Sanchez-Monge and G. Salcedo, technical assistance from D. Lamoneda and J. García, and support from the *Comisión Interministerial de Ciencia y Tecnología* (grant no. PB92-0325) are gratefully acknowledged.

REFERENCES

1. Bowles, D. J. (1990) *Annu. Rev. Biochem. 59*, 873–907.
2. García-Olmedo, F., Salcedo, G., Sánchez-Monge, R., Gómez, L., Royo, J. & Carbonero, P. (1987) *Oxf. Surv. Plant Mol. Cell Biol. 4*, 275–334.
3. García-Olmedo, F., Rodríguez-Palenzuela, P., Hernández-Lucas, C., Ponz, F., Maraña, C., Carmona, M. J., López-Fando, J., Fernández, J. A. & Carbonero, P. (1989) *Oxf. Surv. Plant Mol. Cell Biol. 6*, 31–60.
4. García-Olmedo, F., Carmona, M. J., López-Fando, J. J., Fernández, J. A., Castagnaro, A., Molina, A., Hernández-Lucas, C. & Carbonero, P. (1992) in *Plant gene research series: genes involved in plant defense* (Boller, T. & Meins, F. eds) pp. 283–302, Springer-Verlag, Berlin.
5. Colilla, F. J., Rocher, A. & Méndez, E. (1990) *FEBS Lett. 270*, 191–194.
6. Méndez, E., Moreno, A., Colilla, F., Pelaez, F., Limas, G. G., Méndez, R., Soriano, F., Salinas, M. & Haro, C. D. (1990) *Eur. J. Biochem. 194*, 533–539.
7. Bloch Jr, C. & Richardson, M. (1991) *FEBS Lett. 279*, 101–104.

8. Stiekema, W. J., Heidekamp, F., Dirkse, W. G., van Beckum, J., de Haan, P., ten Bosch, C. & Louwersee, J. D. (1988) *Plant Mol. Biol. 11*, 255–269.
9. Chiang, C. C. & Hadwiger, L. A. (1991) *Mol. Plant-Microbe Interact. 4*, 324–331.
10. Gu, Q., Kawata, E. E., Morse, M-J., Wu, H-M. & Cheung, A. Y. (1992) *Mol. Gen. Genet. 234*, 89–86.
11. Choi, Y., Choi, Y. D. & Lee, J. S. (1993) *Plant Physiol. 101*, 699–700.
12. Ishibashi, N., Yamauchi, D. & Minamikawa, T. (1990) *Plant Mol. Biol. 15*, 59–64.
13. Terras, F. R. G., Schoofs, H. M. E., De Bolle, M. F. C., Van Leuven, F., Rees, S. B., Vanderleyden, J., Cammue, B. P. A. & Broekaert, W. F. (1992) *J. Biol. Chem. 267*, 15301–15309.
14. Terras, F. R. G., Torrekens, S., Van Leuven F., Osborn, R. W., Vanderleyden, J., Cammue, B. P. A. & Broekaert, W. F. (1993) *FEBS Lett. 316*, 233–240.
15. Molina, A., Segura, A. & García-Olmedo, F. (1993) *FEBS Lett. 316*, 119–122.
16. Díaz, I., Carmona, M. J. & García-Olmedo, F. (1992) *FEBS Lett. 296*, 279–282.
17. Bernfeld, P. (1955) in *Methods Enzymol.* (Colowick, S. P. & Kaplan N. O. eds) vol. 1, pp. 149–158, Academic Press Inc., Publishers, NY.
18. Boisen S. & Djurtoft R. (1981) *Cereal Chem. 58*, 194–198.
19. Broekaert, W. F., Terras, F. R. G., Cammue, B. P. A. & Vanderleyden, J. (1990) *FEMS Microbiol. Lett. 69*, 65–60.
20. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1982) *Molecular cloning: a laboratory manual*, 2nd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
21. Church, G. & Gilbert, W. (1984) *Proc. Natl Acad. Sci. USA 81*, 1991–1995.
22. McClure, B. A. & Guilfoyle, T. J. (1989) *Plant Mol. Biol. 12*, 517–524.
23. Varner, J. E. & Cassab, G. I. (1987) *J. Cell Biol. 105*, 2581–2588.
24. Kunoh, H. (1987) in *Molecular determinants of plant diseases* (Nishimura, S., ed.) pp. 59–73, Japan Sci. Soc. Press, Tokyo/ Springer-Verlag, Berlin.
25. Jakobek, J. L., Smith, J. A. & Lindgren, P. B. (1993) *The Plant Cell. 5*, 57–63.