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Purification and antipathogenic activity of lipid transfer proteins (LTPs) from the leaves of *Arabidopsis* and spinach

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Two homogeneous proteins active in vitro against the bacterial pathogen *Clavibacter michiganensis* subsp. *sepedonicus* were obtained from a crude cell-wall preparation from the leaves of Columbia wild-type *Arabidopsis*. The N-terminal amino acid sequences of these proteins allowed their identification as lipid transfer proteins (LTP-a1, LTP-a2); the LTP1-a1 sequence was identical to that deduced from a previously described cDNA (EMBL M80566) and LTP-a2 was quite divergent (44% identical positions). These proteins were not detected in the cytoplasmic fraction by Western-blot analysis. Proteins LTP-s1 and LTP-s2 were similarly obtained from spinach leaves; LTP-s1 was 91% identical to a previously purified spinach LTP (Swiss Prot P10976), and LTP-s2 was moderately divergent (71% identical positions). About 1/3 of the total LTPs were detected in the cytoplasmic fraction from spinach by Western-blot analysis. Concentrations of these proteins causing 50% inhibition (EC-50) were in the 0.1–1 μM range for the bacterial pathogens *C. michiganensis* and *Pseudomonas solanacearum* and close to 10 μM for the fungal pathogen *Fusarium solani*.

Arabidopsis; Lipid transfer protein; Plant pathogen; Spinach; Thionin

1. INTRODUCTION

Various isoforms of the so-called non-specific lipid transfer proteins (LTPs) from leaves of barley and maize have been recently shown to be potent growth inhibitors of bacterial and fungal plant pathogens [1,2]. A defense role for these proteins is further supported by evidence of their preferential cell-wall location in epidermal cells throughout the plant and by the increased expression of their genes in response to pathogens [3]. A defense role is not necessarily incompatible with other possible functions, such as lipid exchange between cell organelles in the cytoplasm [4–6] or cutin biosynthesis [7,8], and it is becoming increasingly evident that a number of widely divergent LTP subfamilies may coexist in a given plant [2,9], which raises the possibility of a certain degree of functional specialization among these subfamilies. A cytoplasmic role, such as lipid exchange between organelles, would be incompatible with a non-cytoplasmic location of the proteins [2,10], so it is of interest to investigate whether LTPs can be also recovered in the soluble fraction. We report here the isolation of LTP isoforms from *Arabidopsis thaliana* and from spinach. All these LTPs were potent growth inhibitors of plant pathogens and were preferentially extracted from crude cell-wall preparations, but about one third of the spinach LTPs were recovered from the soluble fraction.

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2. MATERIALS AND METHODS

2.1. *Biological materials*

Columbia wild-type of *Arabidopsis thaliana* was grown for 24 days, at 22°C, 65% humidity, 18 h light and 6 h dark. Leaf material was collected and immediately frozen in liquid nitrogen. An antisense line (LTP4), with decreased expression of the messenger encoding protein LTP-a1, and rabbit antibodies raised against an LTP:MalE fusion protein which had been produced in *Escherichia coli* [10] were the kind gift of S. Thoma and C. Somerville (East Lansing, Michigan, USA). Frozen Frudesa spinach leaves were also used. Bacterial pathogens *Clavibacter michiganensis* subsp. *sepedonicus*, strain C5, and *Pseudomonas solanacearum*, strain P2, and the fungal pathogen *Fusarium solani*, strain 1, were from the ETSIA collection (Madrid).

2.2. *Purification and characterization of the proteins*

Frozen leaf material (20 g) was ground to powder in liquid nitrogen, using a mortar and pestle, and extracted once with 80 ml of buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5) and twice with 80 ml of distilled H_2O. The resulting pellet was then extracted with 50 ml of 1.5 M LiCl at 4°C for 1 h, dialyzed against 5 l of H_2O, using a Spectra/Por 6 (MWCO:3000) membrane, and freeze-dried. The extract was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on an ultrapore C3 column (1 cm × 25 cm; 5 μm particle; 300 Å pore) from Beckman, using a H_2O/2-propanol gradient, 0.1% trifluoroacetic acid, at 0.5 ml/min. Fractions were collected by hand and freeze-dried. The soluble proteins in the extracts obtained with the initial buffer and with H_2O were pooled, precipitated with saturated ammonium sulfate, and redissolved in electrophoresis sample buffer.

Western-blot experiments were carried out with the pooled soluble proteins and with the salt-extracted fraction, as well as with the purified proteins, after electrophoresis and transfer to PVDF membranes following the manufacturers instructions (Immobilon, Millipore). The first antibody was used at a 1/500 dilution and the second (antirabbit) antibody, coupled to alkaline phosphatase (Sigma), at a 1/5,000 dilution. Visualization was with Nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (Sigma).

The proteins were subjected to sodium-dodecyl-sulfate-polyacrylamide-electrophoresis (SDS-PAGE) in preformed gradient gels

(4–20%; Bio-Rad) according to the manufacturers instructions. Amino acid sequencing was carried out by automated Edman degradation of the intact proteins.

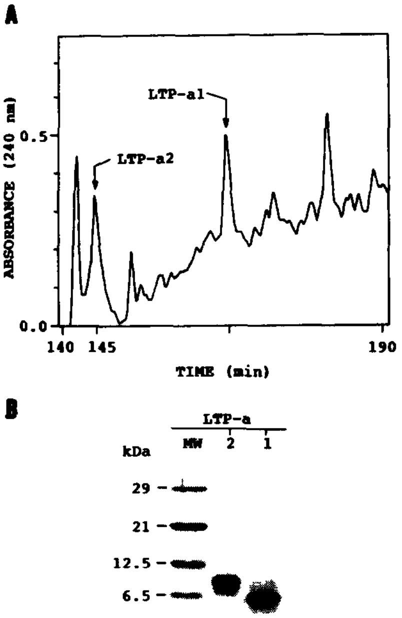
2.3. *Inhibition tests*

Proteins were dissolved in water as required. Bacteria were inoculated at a final concentration of 10^4 cfu/ml in sterile microtiter plates at final volumes of 150 μl (100 μl protein + 50 μl nutrient broth, Oxoid). After 16–24 h of incubation at 28°C, growth was recorded by measuring absorbance at 490 nm in an ELISA plate reader. In the case of the fungus, spores were collected from 8-day-old cultures grown at 25°C on potato dextrose agar plates (Difco) and stored at −20°C in 20% glycerol [11]. Spore suspensions (10^4/ml) were incubated in microtiter plates with the indicated amounts of added proteins (final volume 75 μl) at 25°C for 26–44 h and growth was recorded as above. The thionin used as positive control in inhibition experiments was a mixture of α- and β-thionins from wheat endosperm (gift of A. Molina, Madrid).

3. RESULTS

A crude cell-wall preparation from the leaves of *Arabidopsis thaliana* was obtained by washing twice with H_2O the pellet obtained after centrifugation of the initial buffer homogenate. This preparation was extracted with 1.5 M LiCl and the extract fractionated by RP-HPLC, as shown in Fig. 1A. The protein fractions were screened for their ability to inhibit in vitro growth of the pathogen *C. michiganensis* (at 100 μg/ml) and subjected to SDS electrophoresis. Two electrophoretically-homogeneous fractions that completely inhibited growth at the tested concentration were found (Fig. 1A,B). The N-terminal amino acid sequences of these proteins did not present heterogeneity and were homologous to previously described LTPs. One of the sequences, designated LTP-a1, was identical to that previously deduced from a cloned cDNA [10], and the second sequence, designated LTP-a2, was quite divergent (44% identical residues), but had the conserved features of an LTP N-terminus (Fig. 1C). The yields of these proteins were in the range 5–10 mg/kg fresh weight.

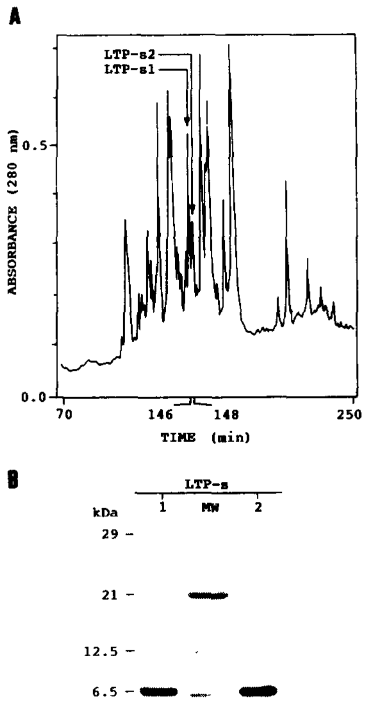
The same purification procedure was applied to spinach leaves and two homogeneous proteins were obtained (Fig. 2A,B), one of which, designated LTP-s1 (Fig. 2C), was almost identical (91% identical residues at the N-terminus) to a previously purified spinach LTP [12], and the other (LTP-s2; 71% identical positions) presented moderate divergence with respect to that pro-



C

cDNA	ALSCGSVNSSNLAACIGYV
a1	ALSCGSVNSXNXA
a2	AISCGAVTGSLGQ.CYNYL

Fig. 1. Purification of proteins LTP-a1 and LTP-a2, (A) RP-HPLC fractionation of the 1.5 M LiCl extract from the insoluble pellet from a Tris-HCl (pH 7.5) homogenate from the leaves of *Arabidopsis*. The gradient used was H_2O (0.1% trifluoroacetic acid)-2-propanol linear 0–30% for 180 min, followed by linear 30–50% for 15 min. (B) Separation by SDS-PAGE of the purified proteins. Molecular mass markers (MW) were carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), cytochrome *c* (12.5 kDa), and bovine lung trypsin inhibitor (6.5 kDa). (C) N-terminal amino acid sequences aligned with the sequence deduced from a previously reported *Arabidopsis* LTP cDNA (EMBL M80566).



C

cDNA	GITCGMVSSKLAPCIGYLKGGPL
s1	GITCGNVSSKLAPCIGYLNGGPL
s2	GITCAMVARAPAPCIGYLIGG

Fig. 2. Purification of proteins LTP-s1 and LTP-s2, (A) RP-HPLC fractionation of spinach leaf extract as in Fig. 1. (B) SDS-PAGE of purified proteins; molecular mass (MW) standards as in Fig. 1. (C) N-terminal amino acid sequences of purified proteins aligned with that deduced from a previously reported [14] LTP cDNA from spinach.

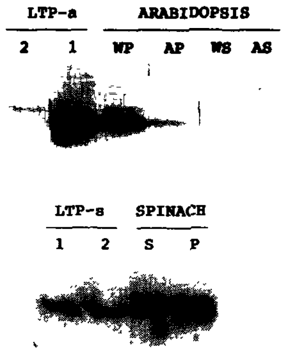


Fig. 3. Western-blot analysis of the cytoplasmic fraction (supernatant of the initial low-salt homogenate, S) and the cell wall fraction (high-salt extract from pellet, P) from leaves of Columbia wild-type (W) and antisense (A) *Arabidopsis* plants and from leaves of Frudesa spinach.

tein (Fig. 2C). Yields were in the range 1–2 mg/kg fresh weight.

Antibodies raised against an *Arabidopsis* LTP-a1 fusion protein produced in *Escherichia coli* recognized the isolated LTPs from both species (Fig. 3A,B). In *Arabidopsis*, the antiserum detected the LTPs in the salt extract from the crude cell wall preparation and not in the soluble fraction (supernatant of the initial low-salt homogenate), both in the wild-type and in the antisense plants (Fig. 3A). In the antisense plants, LTP-a1 was decreased to about 40% of the wild-type level, whereas LTP-a2 was not affected. In spinach, the same antiserum seemed to detect not only LTP-s1 and LTP-s2, which had the same electrophoretic mobility, but also additional, slower-moving LTPs, and about 1/3 of all the detected LTPs appeared in the soluble fraction (Fig. 3B).

Growth inhibition by the four purified LTPs was tested against two bacterial and one fungal pathogen, using a mixture of wheat thionins as positive control (Fig. 4). The spinach LTPs were about tenfold more effective than the *Arabidopsis* LTPs or the thionin control against *C. michiganensis*; LTP-s1 was about fivefold more active than the rest against *P. solanacearum*; and there was no significant difference between any of the tested proteins and the thionin control against *F. solani*.

4. DISCUSSION

A simple, two-step procedure allowed the purification of LTPs from washed pellets of the leaves from *Arabidopsis* and spinach, which was in line with the proposed preferential location of LTPs in the cell wall [2,3,8,10] and indicated the effectiveness of the purification method in dicots as well as in monocots [2]. In spinach, but not in *Arabidopsis*, up to 1/3 of the LTPs appeared in the cytoplasmic fraction, which suggested that either the association of these proteins with the cell wall was more labile in that species, or that these proteins might also have a cytoplasmic location. This was also in agreement with the previous report of a spinach LTP isolated from the cytoplasmic fraction, which closely resembled LTP-s1 [12].

These observations also confirmed that multiple LTP isoforms are present in plants and that the ability to inhibit growth of bacterial and fungal plant pathogens is a general property of LTPs [1,2,13]. Naturally occurring LTP concentrations in the two species under study are within the same order of magnitude of those required to cause inhibition in vitro. The bacterial pathogens were generally more sensitive to LTPs and to thionin than the fungal one. More variation in the effectiveness of the different LTPs was observed for certain pathogens, such as *C. michiganensis*, than for others.

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REFERENCES

[1] Molina, A. and García-Olmedo, F. (1991) Patent application P 9101258 (24.05.91) PCT/EP92/01130.

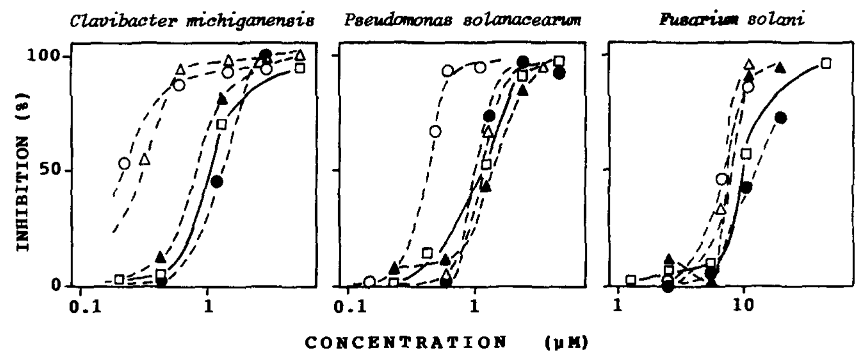

Fig. 4. In vitro inhibition tests with the indicated plant pathogens. The following symbols are used LTP-a1 (●), LTP-a2 (▲), LTP-s1 (○), LTP-s2 (△), $\alpha 1+\beta$ thionin from wheat (□).

[2] Molına, A., Segura, A. and García-Olmedo, F. (1993) FEBS Lett. 316, 119–122.
[3] Molina, A. and García-Olmedo, F. (1993) Plant J., in press.
[4] Breu, V., Guerbette, F., Kader, J.C., Kannangara, C.G., Svensson, B. and Wettstein-Knowles, P. (1989) Carlsberg Res. Commun. 54, 81–84.
[5] Arondel, V., Kader, J.-C. (1990) Experientia 46, 579–585.
[6] Watanabe, S. and Yamada, M. (1986) Biochim. Biophys. Acta 876, 116–123.
[7] Hendricks, T., Meijer, E.A., Thoma, S., Kader, J.C. and De Vries, S.C. (1993) NATO/ASI Workshop on Plant Molecular Biology (G. Coruzzi and P. Puigdomenech, Eds.) Springer, NY, in press.
[8] Sterk, P., Booij, H., Schellekens, G.A., van Kammen, A. and De Vries, S.C. (1991) Plant Cell 3, 907–921.
[9] Takishima, K., Watanabe, J., Yamada, M., Suga, T. and Mamiya, G. (1988) Eur. J. Biochem. 177, 241–249.
[10] Thoma, S., Kaneko, Y. and Somerville, C. (1993) Plant J. 3, 427–436.
[11] Brockaert, W.F., Terras, F.R.G., Cammue, B.P.A. and Vanderleyden, J. (1990) FEMS Microbiol. Lett. 69, 55–60.
[12] Bouillon, P., Drischel, C., Vergnolle, C., Duranton, H. and Kader, J.C. (1987) Eur. J. Biochem. 166, 387–391.
[13] Franky, R.G., Terras, I.J.G., Van Leuven, F., Vanderleyden, J., Cammue, B.P.A. and Brockaert, W.F. (1992) Plant Physiol. 100, 1055–1058.
[14] Bernhard, W.R., Thoma, S., Botella, J. and Somerville, C.R. (1991) Plant Physiol. 95, 164–170.