

Purification, characterization and antifungal properties of a lipid-transfer protein from sunflower (*Helianthus annuus*) seeds

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An antifungal protein from *Helianthus annuus* L. seeds (Ha-AP10) has been purified to homogeneity and characterized. Ha-AP10 purification was performed by gel filtration, cation exchange chromatography and reverse phase HPLC. Its molecular mass was estimated to be 10 kDa and western blot analyses suggest that it has an extracellular location. The N-terminal sequence of Ha-AP10 showed strong homology to

some plant lipid-transfer proteins (LTPs). Antifungal tests have demonstrated that Ha-AP10 exerts a fungistatic effect. It completely inhibits the germination of spores of the fungal pathogen *Fusarium solani* f. sp. *eumartii* at a concentration of $40 \mu\text{g ml}^{-1}$ and produces a 50% growth inhibition at $6.5 \mu\text{g ml}^{-1}$ ($0.65 \mu\text{M}$). These data place Ha-AP10 among the most potent antifungal LTPs described so far.

Introduction

Antimicrobial proteins are ubiquitously produced throughout nature to protect organisms against microbial attack (Boman 1995). During the last decade, several plant proteins capable of inhibiting fungal growth in vitro have been isolated and characterized. Among them are glucanases and chitinases (Mauch et al. 1988), thaumatin-like proteins (Hejgaard et al. 1991, Vigers et al. 1991, Woloshuk et al. 1991) and several kinds of basic cysteine-rich peptides (Broekaert et al. 1997). The latter group includes thionins (García Olmedo et al. 1989, Bohlmann and Apel 1991), lipid-transfer proteins (LTPs) (Terras et al. 1992, Molina et al. 1993), defensins (Broekaert et al. 1995), and other less-known low molecular weight proteins. These peptides can inhibit a wide variety of phytopathogenic fungi but display different potencies according to the target microorganism.

By now, the only role proposed for thionins and defensins is plant defense. On the other hand, the in vivo role of plant LTPs is still controversial, with experimental evidences supporting the different proposed functions (Kader 1997). Plant LTPs of about 9–10 kDa were originally thought to be involved in lipid shuttling between organelles because of their ability to transfer lipids between membranes in vitro (Arondel and Kader 1990, Kader 1996). However, a body of data now exists indicating that LTPs are synthesized as

precursors and are frequently found associated with the cell wall and secreted in cell cultures. Sterk et al. (1991) have suggested that they may be involved in cuticle formation and deposition of other extracellular lipophilic compounds. More recently also, a defensive role of LTPs has been proposed considering the ability of some members of this family, but not all, to inhibit the growth of bacterial and fungal pathogens in vitro (García Olmedo et al. 1995). However, both hypotheses on the role of LTPs are not contradictory and, indeed, the characterization of distinct members of the LTP family is suggesting that multiple genes may play multiple roles in vivo (see Kader 1997 for review).

Several studies suggest that antimicrobial peptides may have a direct antimicrobial activity in vivo, especially the enhanced resistance to microbial pathogens conferred to transgenic plants over-expressing thionins (Carmona et al. 1993, Holtorf et al. 1998), a defensin (Terras et al. 1995) or an LTP (Molina and García Olmedo 1997). In addition, their constitutive expression and external localization in some plant organs has been described (see Broekaert et al. 1997 for review), suggesting a potential contribution to the innate immunity, which refers to the first-line defense, limiting infection in the early hours of microbial attack. The concept of innate immunity in plants is becoming relevant

Abbreviations – FABP, fatty acid-binding protein; Ha-AP10, *Helianthus annuus* antifungal protein 10 kDa; LIS, low ionic strength; LTPs, lipid-transfer proteins; PVDF, polyvinylidene difluoride; RP, reverse phase; TFA, trifluoroacetic acid.

after the reappearance of the subject as a central defense mechanism in mammals and insects (Hoffmann et al. 1999). In fact, unlike phytoalexins, which can have adverse effects in plants, antifungal peptides do not seem to be phytotoxic and can attain high levels in seeds, where they are believed to play a role as protectants.

In the present work we looked for antifungal peptides of constitutive expression in sunflower seeds and we describe the characterization of a potent antifungal lipid-transfer protein.

Materials and methods

Biological material

Seeds of sunflower (*Helianthus annuus* L.) line AR10018 (Zeneca SAIC, Centro Biotecnológico Balcarce, Argentina) were used in this study. *Fusarium solani* f. sp. *eumartii*, isolate 3122, (INTA Collection, Balcarce, Argentina) was kindly provided by Dr C. A. Casalengué and colleagues. The fungus was grown at 25°C on potato dextrose agar plates and spores were collected from 8-day-old cultures by suspension in sterile water.

Antifungal activity assays

The microscopic evaluation of the inhibition of *F. solani* spore germination was carried out on micro slides in a final volume of 25 µl containing the protein sample (in water or sodium acetate 50 mM, pH 5.2), 1.5×10^3 spores and 4% (w/v) sucrose. Controls were performed replacing the protein sample with the same volume of water or buffer. After 16 h of incubation at 25°C and at a 100% relative humidity, samples were microscopically evaluated for the presence of germinated spores. A quantitative test was performed in flat microtiter plates containing the protein sample to be tested (in water) and 0.5×10^3 spores in a low ionic strength (LIS) medium (Cammue et al. 1992), in a final volume of 50 µl. Samples were incubated at 25°C for 2 days and fungal growth was monitored at 550 nm in an ELISA plate reader.

Protein purification

Dried seeds (50–200 g) were homogenized in four volumes of extraction buffer (sodium acetate 100 mM, KCl 100 mM, 0.1% β -mercaptoethanol, pH 5.2) and extracted overnight at 4°C. The extract was filtered and centrifuged at 10000 g for 20 min. The soluble fraction was then precipitated with ammonium sulfate at 80% of saturation. The pellet was collected by centrifugation at 10000 g for 30 min, suspended in water, and finally heated at 80°C for 15 min. The soluble fraction obtained after centrifugation at 10000 g for 10 min was submitted to gel filtration on a Biogel P10 column equilibrated in sodium acetate 50 mM, pH 5.2. The low molecular mass fractions displaying antifungal activity were pooled and subjected to cation exchange chromatography on a CM-Sepharose column equilibrated with 20 mM sodium acetate pH 5.2. Bound

proteins were eluted using 0.1, 0.2, 0.3 and 0.5 M NaCl and monitored at 280 nm. The protein peak eluted at 0.2 M NaCl was dialyzed (membrane cut-off 2000 Da) against water and submitted to reverse phase (RP) HPLC on a C₈ 5 µm column (Microsorb-MV; Rainin Instrument Company, Woburn, MA, USA) equilibrated with water containing 0.1% trifluoroacetic acid (TFA). The following gradient (at a flow rate of 1 ml min⁻¹) was applied: 0–10 min, 0.1% TFA; 10–50 min, 0–50% acetonitrile in 0.1% TFA; 50–55 min, 50–100% acetonitrile in 0.1% TFA; 55–57 min, 100% acetonitrile in 0.1% TFA; 57–60 min, 100–0% acetonitrile in 0.1% TFA; 60–75 min, 0.1% TFA. Fractions were collected by hand, dried on speed-vac and redissolved in water twice to remove the TFA before determination of the antifungal activity. The positive fractions were submitted to a second RP-HPLC on a C₁₈ 5 µm Jupiter column (Phenomenex, Torrance, CA, USA) equilibrated in 0.1% TFA. The following elution (at a flow rate of 1 ml min⁻¹) was performed: 0–10 min, 0.1% TFA; 10–15 min, 0–15% acetonitrile in 0.1% TFA; 15–75 min, 15–45% acetonitrile in 0.1% TFA; 75–80 min, 45–100% acetonitrile in 0.1% TFA; 80–82 min, 100% acetonitrile in 0.1% TFA; 82–85 min, 100–0% acetonitrile in 0.1% TFA; 85–95 min, 0.1% TFA. Peak fractions were dried on speed-vac and suspended in water (generally twice) until the pH of the sample was that of water, indicating the absence of residual TFA, which could interfere with further analyses. The purity of the sample obtained, named Ha-AP10, was evaluated under different RP-HPLC running conditions and SDS-PAGE followed by silver nitrate staining.

Protein analysis

Protein concentration was determined using the bicinchoninic acid assay (Smith et al. 1985) using bovine serum albumin (BSA) as standard. Electrophoretic separation was performed in 15% SDS-PAGE gels (Laemmli 1970), and stained with silver reagent. For N-terminal sequencing analysis of Ha-AP10, after electrophoretic migration the sample was transferred to a PVDF membrane using a semi-dry apparatus (Bio-Rad, Melville, NY, USA), the blot was stained with Coomassie blue and the protein band was cut off. Prior to N-terminal sequencing (Lanaís-Pro, Buenos Aires, Argentina) the sample was treated with 0.6 M HCl for 24 h at 25°C and N-terminal amino acid sequence was determined by automatic Edman degradation in a 477 Protein Sequenator (Applied Biosystems, Foster City, CA, USA). Protein sequence homology was analyzed using the BLAST database search (<http://www.ncbi.nlm.nih.gov>).

Antibody production

An homogeneous fraction of Ha-AP10 was used to raise antibodies in mice. The anti-Ha-AP10 antibody obtained was used at a 1/10000 dilution and a second antibody coupled to alkaline phosphatase (Sigma, St Louis, MO, USA) was used to visualize the signals with Nitroblue Tetrazolium (Sigma) and 5-bromo-4-indolylphosphate disodium salt (Sigma).

Western blot analysis

Total extracts from seeds were obtained with the TRIzol Reagent (GIBCO-BRL, Gaithersburg, MD, USA) as described by Chomczynski (1993). Washing extracts were obtained by washing the intact material with 3 volumes of extraction buffer (1.5 M LiCl, 0.01% SDS) for 15 min. Samples were then centrifuged at 2000 g for 5 min, and the clarified extract was precipitated with acetone. The protein was resuspended in electrophoresis sample buffer and fractionated on 15% SDS-PAGE. Western blot experiments were carried out after transfer of the protein samples to nitrocellulose membranes.

Results

Purification of an antifungal peptide from sunflower seeds

We have analyzed sunflower seed extracts for the presence of antifungal peptides by testing their ability to inhibit the germination of *F. solani* spores. A heat-resistant low molecular weight protein fraction was obtained and then submitted to cation exchange chromatography, where several antifungal proteins were eluted. The basic fraction eluting at 0.2 M NaCl was pooled and further purified by RP-HPLC (Fig. 1A). The major peak showing antifungal activity was submitted to a second RP-HPLC and eluted with a more expanded gradient. Fig. 1B shows this antifungal protein (Ha-AP10) as a sharp peak, which, in a subsequent analysis on SDS-PAGE followed by silver staining, reveals a single detectable band with an apparent molecular mass of 10 kDa (Fig. 1B, inset).

Ha-AP10 is homologous to LTPs

The first 20 amino acids of the purified peptide Ha-AP10 were determined by automated Edman degradation (Fig. 2). By comparing its N-terminal sequence with those available in data banks, we found that it was identical to a previously described bifunctional lipid-transfer:fatty acid-binding protein (LTP-FABP) from sunflower (Arondel et al. 1990) and homologous to potential lipid-transfer proteins (LTPs) deduced from cDNA clones (Fig. 2). The most homologous protein sequences were those LTPs from tobacco and carrot, which show an identity of 68 and 61%, respectively. The alignment of Ha-AP10 with these LTPs reveals the presence of some conserved residues supposed to be essential for their structure (Broekaert et al. 1997). As noted in Fig. 2, these conserved residues in the mature protein are: cysteine at positions 3 and 13, valine at position 6, a hydrophobic residue (leucine) at position 14 and an aromatic residue (tyrosine) at position 16. In addition, good conservation of several hydrophobic residues (I, L, P) is observed. The sequence of another sunflower LTP, named SDi-9, deduced from a cDNA clone differentially expressed under drought conditions has already been described (Ouvrard et al. 1996). However, except for the consensus sequence, no extensive homology is seen in the N-terminal domain among both sunflower LTPs (Fig. 2).

Antifungal activity of Ha-AP10

The effect of a pure fraction of Ha-AP10 on the germination of *F. solani* spores upon microscopic evaluation is shown in Fig. 3. After overnight incubation, Ha-AP10 completely inhibited the germination of *F. solani* at a concentration of 40 $\mu\text{g ml}^{-1}$ producing no apparent cell lysis. In order to determine if Ha-AP10 was also able to control hyphal growth once the spores have germinated, the activity of Ha-AP10 on *F. solani* mycelial growth was evaluated by measuring fungal biomass at 550 nm. In vitro quantitative assays showed that the growth inhibition produced by Ha-AP10 is dose dependent (Fig. 4A), displaying an estimated IC_{50} value (concentration required to produce a 50% growth inhibition) of approximately 6.5 $\mu\text{g ml}^{-1}$ (0.65 μM). To investigate whether Ha-AP10 produces a lethal effect against *F. solani*, cultures of this fungus were grown in the presence of 25 $\mu\text{g ml}^{-1}$ of the protein and after 3 days of incubation, when these cultures were inhibited by 80% compared with controls without Ha-AP10, duplicate cultures

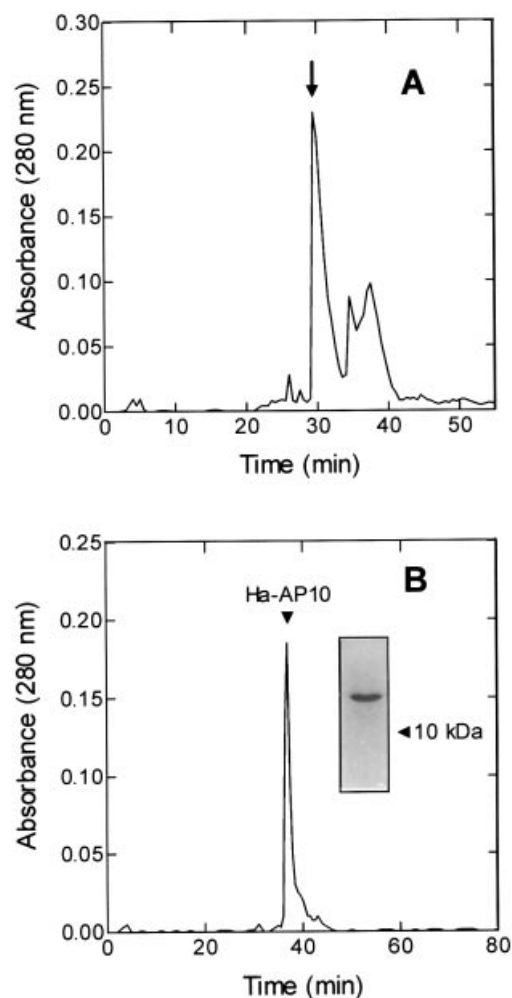


Fig. 1. Purification of sunflower protein Ha-AP10. A: C_{18} RP-HPLC fractionation of the heat-resistant low molecular mass basic proteins from sunflower seeds. The arrow indicates the major peak with antifungal activity. B: The peak marked in A was further purified by chromatography with a expanded gradient on C_{18} RP-HPLC. Inset: SDS-PAGE analysis of Ha-AP10.

		--C--V-----Cf-a----	
Ha-AP10	:	ITQNDVTGNITFCIPVLRSS	: 20
LTP-FABP	:	ITQNDVTGNITFCIPVLRSS	: 20
N-LTP1	:	ITGGQVTSNIAFCIAYLRNT	: 20
EP2	:	LITGGQVTGAAFCIGVLRSQ	: 20
Le-nsLTP	:	LITGGQVTAGAAFCIPVLRQGR	: 20
SDi-9	:	LSTGGQVSSRIAFGISLTKG	: 20

Fig. 2. Alignment of the N-terminal amino acid sequence of Ha-AP10 with homologous LTPs. The following N-terminal sequences are included: LTP-FABP, bifunctional lipid-transfer:fatty acid-binding protein from sunflower (Arondel et al. 1990); N-LTP1 from *Nicotiana tabacum* (Fleming et al. 1992); EP2 from *Daucus carota* zygotic embryos (Sterk et al. 1991); Le-nsLTP from *Lycopersicon esculentum* (Torres-Schumann et al. 1992); SDi-9 sunflower drought-induced nsLTP (Ouvrard et al. 1996). Conserved residues are presented in black boxes. The LTP consensus sequence is shown above the alignment (a: aromatic amino acids; f: hydrophobic amino acids).

were supplemented with LIS medium. Fig. 4B shows that, in the presence of added medium, cultures started their growth while plates which were not supplemented with LIS medium remained inhibited. These results suggest that Ha-AP10 at the concentration tested is not acting as a fungicide, but instead, has a fungistatic effect.

Location of Ha-AP10

We performed western blot assays to analyze if Ha-AP10 has an extracellular location. A polyclonal antibody raised against Ha-AP10 recognized the protein in seed homogenates as well as in washing extracts obtained by simple dipping of decoated dried seeds in a high salt solution (Fig. 5). A faint signal was also detected in seed coat washing extracts (Fig. 5) and was clearly visible in blots containing 40 mg fresh weight per lane (not shown). These results indicate that the protein is accumulated in the apoplast at an external location, although it cannot be excluded that a minor part could be released from a putative intracellular fraction by wounding of peripheral cells during decoating.

Discussion

A basic 10 kDa protein exerting antifungal activity (Ha-AP10) has been purified to homogeneity from *H. annuus* seeds. According to its properties and the sequence of its 20 N-terminal amino acids, Ha-AP10 appears to be a member

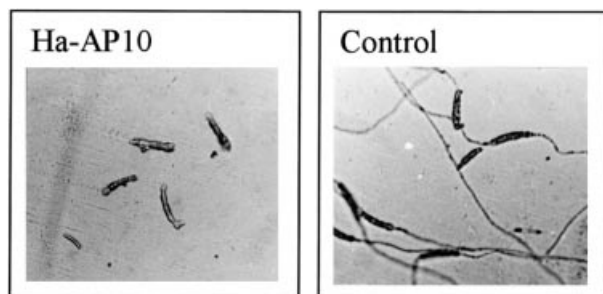


Fig. 3. Morphology of *F. solani* spores grown for 16 h in the presence of $40 \mu\text{g ml}^{-1}$ of Ha-AP10 (left panel) or in its absence (right panel). Magnification $150\times$.

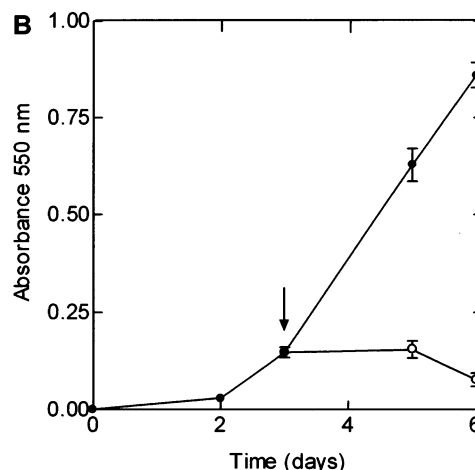
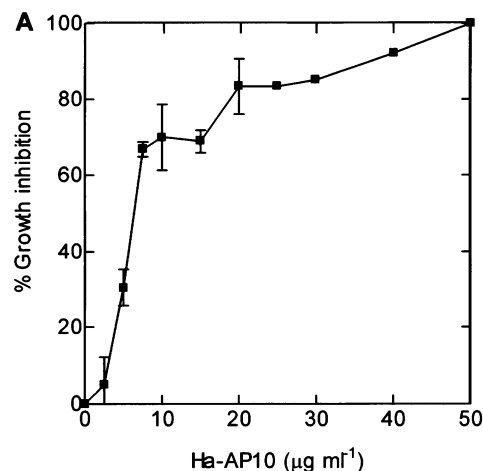


Fig. 4. A: Growth inhibition of *F. solani* in the presence of different concentrations of Ha-AP10. Growth of *F. solani* in LIS medium without Ha-AP10 was taken as 100%. B: Fungistatic effect of Ha-AP10. After 3 days of incubation in the presence of $25 \mu\text{g/ml}$ of Ha-AP10, some cultures were supplemented with $50 \mu\text{l}$ of LIS medium (●), whereas the others remained with the initial medium (○). The addition of medium is indicated with an arrow.

of the family of LTPs. In fact, this sequence is identical to the N-terminal domain of a previously described bifunctional lipid-transfer:acyl binding protein (LTP-FABP) isolated from sunflower seedlings by its ability to transfer

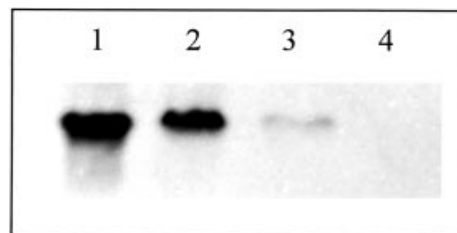


Fig. 5. Extraction of Ha-AP10 from intact seeds. Protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose and immunodetected with anti-Ha-AP10 antibodies. Lane 1, homogeneous Ha-AP10, Lane 2, seed homogenate (3 mg FW). Lane 3, washing extract from decoated seeds (30 mg FW), Lane 4, washing extract from seed coat (30 mg FW).

phosphatidylcholine between membranes and to bind oleoyl-CoA in vitro (Arondel et al. 1990). As Ha-AP10, LTP-FABP is also a basic protein with an apparent molecular mass of 9 kDa on SDS-PAGE. However, the data available do not allow us to conclude whether the bifunctional LTP-FABP from seedlings is the same protein isolated from seeds. Furthermore, another basic protein with LTP activity was detected, but not characterized, in sunflower seedlings (Arondel et al. 1990). As LTPs are generally encoded by a small multigenic family (Kader 1996), individual proteins with minor differences may have specific localizations and perform different functions (Clark and Bohnert 1999).

Some LTPs with antimicrobial activity have been described in monocots and a few cases have been reported in dicotyledonous plants, namely in members of Brassicaceae and Quenopodiaceae. The description of Ha-AP10 in sunflower, a member of the Asteraceae family, extends the distribution of antifungal LTPs among dicots. Moreover, the antifungal tests performed have demonstrated a strong inhibitory activity of Ha-AP10 on the mycelial growth of *F. solani* ($IC_{50} = 6.5 \mu\text{g ml}^{-1}$, i.e., $0.65 \mu\text{M}$). The antimicrobial activity of LTPs seems to be dependent on the target cell and the nature of each protein, showing IC_{50} values varying from $0.25 \mu\text{g ml}^{-1}$ to values greater than $800 \mu\text{g ml}^{-1}$, the latter being considered practically inactive (Cammue et al. 1995, Dubreil et al. 1998). Comparing the antifungal potency of different LTPs against *F. solani*, Ha-AP10 is stronger than the antifungal LTPs isolated from *Arabidopsis* and spinach (Segura et al. 1993) and from barley (Molina et al. 1993) since their IC_{50} values range between 20 and $100 \mu\text{g ml}^{-1}$. This places Ha-AP10 among the most potent antifungal proteins homologous to LTPs described, comparable with those isolated from onion seeds (Cammue et al. 1995) and sugar beet leaves (Nielsen et al. 1996) which display IC_{50} values ranging between 0.25 and $6 \mu\text{g ml}^{-1}$ for other fungi.

Ha-AP10 is also able to restrict *F. solani* spores germination causing a complete inhibition at a concentration of $40 \mu\text{g ml}^{-1}$. The high protein concentration required to abolish the germination may be related to the fact that Ha-AP10 seems to be fungistatic without causing cell lysis. In this sense, it has been reported that a LTP from radish has no effect on the germination of *Botrytis cinerea* at a concentration of $50 \mu\text{g ml}^{-1}$ (Terras et al. 1992). However, these results can be explained by the fact that the concentration of antifungal protein tested was only slightly above its IC_{50} value ($45 \mu\text{g ml}^{-1}$). In fact, according to our results it can be hypothesized that, at sufficiently high protein doses, antifungal LTPs can also inhibit spore germination.

The extracellular location of some LTPs has been demonstrated (Bernhard et al. 1991, Sterk et al. 1991, Thoma et al. 1993). This has been interpreted as supporting a role of these polypeptides in the secretion of lipids into the extracellular space (Kader 1996). However, as most plant fungal pathogens have an extracellular location during the colonization process, antifungal proteins potentially active in vivo are proposed to be located extracellularly. In fact, most antimicrobial peptides have this pattern of expression (Broekaert et al. 1997). Our results show that a substantial

fraction of Ha-AP10 can be extracted by dipping intact decoated seeds into a high-salt buffer, demonstrating that at least part of the protein may be located at an external position. Thus, Ha-AP10 would be accessible to potential pathogens and is a good candidate to be involved in the first-line defense limiting the infection in the early contact with fungi.

It has been reported that constitutive overexpression of the barley LTP2 in transgenic plants of tobacco and *Arabidopsis* enhances tolerance to the bacterial pathogen *Pseudomonas syringae* (Molina and García Olmedo 1997). This evidence strongly supports a role of at least some LTPs in plant defense, although others might have different functions (Tchang et al. 1988, Arondel and Kader 1990, Plant et al. 1991, Sterk et al. 1991, Hughes et al. 1992, Torres-Schumann et al. 1992, Yamada 1992). The results reported in the present work demonstrate a potent inhibition of fungal growth exerted by Ha-AP10. We currently have no direct proof for the linkage between the growth inhibition effect shown in vitro and the activity of Ha-AP10 in planta but according to its potency it could be a good candidate for transgenic overexpression in plants to evaluate enhance protection against fungal pathogens.

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