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Stress induction and antimicrobial properties of a lipid transfer protein in germinating sunflower seeds

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Summary

Nonspecific lipid transfer proteins (nsLTPs) belong to a large family of plant proteins whose function in vivo remains unknown. In this research, we studied a LTP previously isolated from sunflower seeds (Ha-AP10), which displays strong antimicrobial activity against a model fungus. The protein is present during at least the first 5 days of germination, and tissue printing experiments revealed the homogeneous distribution of the protein in the cotyledons. Here we report that Ha-AP10 exerts a weak inhibitory effect on the growth of *Alternaria alternata*, a fungus that naturally attacks sunflower seeds. These data put into question the contribution of Ha-AP10 as an antimicrobial protein of direct effect on pathogenic fungus, and rather suggest a function related to the mobilization of lipid reserves. We also show that the levels of Ha-AP10 in germinating seeds increase upon salt stress, fungal infection and ABA treatment, indicating that it somehow participates in the adaptative responses of germinating sunflower seeds.
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Introduction

Plant nonspecific lipid transfer proteins (nsLTPs) constitute a family of basic polypeptides that are ubiquitous in the plant kingdom (see Kader, 1996, for review). Their primary sequence of 90–95 amino acids presents typical features including conserved positions for eight cysteines and certain hydrophobic residues. The three-dimensional structure of some LTPs has been determined, and a compact structure maintained by four alpha-helices connected by four disulfide bridges has been revealed.

Abbreviations: ABA, *cis*-abscisic acid; Dai, days after inoculation; Dpi, days post imbibition; Ha-AP10, *Helianthus annuus* antifungal protein 10 kDa; LTP, lipid transfer protein; SAR, systemic acquired resistance; SDS, sodium dodecyl sulfate, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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This structure presents a cavity that can accommodate a fatty acid or a lysophospholipid molecule (Kader, 1997). These data have been analyzed concerning a putative function of LTPs in lipid binding and transfer, although no demonstration of such function in vivo has been presented. However, some LTPs can enhance the transfer of lipids between membranes in vitro (Arondel et al., 1990). It has also been suggested that LTPs may participate in the transport of lipids during cuticle formation (Sterk et al., 1991), and in the recycling of lipids from senescing endosperm cells (Edqvist and Farbos, 2002).

On the other hand, the antimicrobial activity described for certain LTPs (Terras et al., 1992; Molina et al., 1993), and the potential participation in the production/transmission of a mobile signal required for SAR (Maldonado et al., 2002), both suggest a role of certain LTPs in plant defense. The analysis of the antimicrobial potency in vitro of several members of the LTP family has shown that they can exert distinct activities. For example, a LTP from onion is highly active against a broad range of fungi, whereas LTPs from maize and wheat seeds are practically inactive (Cammue et al., 1995). The hypothesis of the defensive role in vivo of LTPs has been reinforced by the observation of enhanced resistance to microbial pathogens in transgenic plants overexpressing a LTP gene (Molina and Garcia-Olmedo, 1997). Finally, other experimental evidence indicates that LTPs may be involved in abiotic stress (Kader, 1996) and plant development (Sterk et al., 1991; Fleming et al., 1992). Despite the extensive description of LTPs, their possible role/s in vivo remains open to debate.

Ha-AP10 is a 10 kDa basic polypeptide homologous to many plant LTPs, and originally detected as an antifungal protein present in dried sunflower seeds (Regente and de la Canal, 2000). The presence of a signal peptide in its cDNA (Regente and de la Canal, 2003), and the detection of the protein in the washing fluid (Regente and de la Canal, 2000) indicate the extracellular location of the Ha-AP10 in seeds. The aim of this work was to gain insight on the possible role of Ha-AP10. We analyzed the distribution of the protein, its modulation under stress and its antifungal ability, in order to gain insight into its putative function.

Materials and methods

Biological material

Seeds of sunflower (*Helianthus annuus*) line AR10018 (Zeneca SAIC, Centro Biotecnológico Balcarce, Argentina) were used in this study. The seeds were placed in Petri dishes containing two pieces of filter paper imbibed with sterile distilled water, and then germinated under controlled environmental conditions at 25 °C and under 14-h day cycles, except for infection tests that were performed in the dark. When needed, seedlings were transferred to soil and grown under standard greenhouse conditions to obtain adult plants. Samples from different plant parts were frozen in liquid nitrogen and stored at −70 °C until used.

The fungus *Alternaria alternata* was provided by Dr. C. Cordo (University of La Plata, Argentina), and corresponds to a local isolate. The fungus was grown in the dark at 25 °C and 100% relative humidity on potato dextrose agar plates.

Experimental treatments

Sunflower seeds were imbibed for 16 h, and were then surface sterilized by the following procedure: 1 min in 70% (v/v) ethanol, 10 min in 10% (v/v) commercial hypochlorite solution, and five washes of 5 min in sterile distilled water. To determine the changes in the levels of Ha-AP10 during the germination process, dried seeds and germinating seeds of 0 days post-imbibition (dpi) (immediately after sterilization), 1 dpi, 2 dpi (5 mm long radicles), 3 dpi (10–15 mm long radicles) and 5 dpi (seedlings with 30 mm long radicles, elongated hypocotyles and expanded cotyledons) were harvested. For abiotic stress treatments, germinating seeds (0 dpi) were treated with 300 mM NaCl, 5 μM ABA or sterile water as a control. Experimental and control seeds were harvested 2 days after each treatment. For fungal infection, seeds were inoculated with the fungus *A. alternata* as follows: surface-sterilized seeds were placed equidistant from the growing line of the fungus in Petri dishes containing potato dextrose agar. Mock inoculated seeds were placed in Petri dishes containing only the growth medium. Inoculated and mock inoculated seeds were harvested at different times after inoculation (dai): 0 dai (immediately after placing the seeds in the Petri dish), 1 dai (symptoms of illness were not observed), 2 dai (the mycelium began to grow over the germinating seeds) and 3 dai (the seeds were completely covered by the growing mycelium). After each treatment, 2 seeds were collected, immediately frozen in liquid nitrogen and stored at −70 °C until used.

Protein electrophoresis and immunoblot analysis

Leaves, flowers, hypocotyls, roots, seedlings and seeds were pulverized with liquid nitrogen, and proteins were extracted with 12 mM Tris–HCl pH 6.8, 0.4% v/v SDS using a fresh weight/volume of extraction buffer proportion of 1:3. After 16 h of incubation at 4 °C, insoluble debris were removed by centrifugation at 11,000*g* for 5 min. Protein samples (30 μg lane^{-1}) were fractionated on 15% SDS-PAGE (Laemmli, 1970). Gels were stained with Coomassie Blue or were electroblotted onto 0.22 μm pore size nitrocellulose (semidry procedure). To check the efficiency and evenness of transfer, nitrocellulose was stained for 15 min at room temperature in Red Ponceau (0.1% w/v Red Ponceau, 3% v/v acetic acid). Mouse anti-Ha-AP10 antibodies (Regente and de la Canal, 2000) were used at a 1:8000 dilution to detect Ha-AP10. Protein blots were finally probed with goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma). Nitroblue tetrazolium chloride (Sigma) and 5-bromo-4-chloro-3-indoylphosphate (Sigma) were used as substrate for color development. The signals detected were scanned and then quantified by densitometric analysis with the programme Matrox Inspector 2.2.

Immunolocalization

For tissue print immunolocalization, seeds were cut into halves, and each half was printed on a nitrocellulose membrane (0.22 μm pore size) pre-wetted in buffer 100 mM Tris–HCl, 140 mM NaCl, pH 7.5. Prints were first stained for total protein with Red Ponceau, and then used for immunolocalization of Ha-AP10 as described above for immunoblot analysis.

Antifungal activity assays

Microscopic evaluation of the inhibition of *A. alternata* spore germination was carried out on micro slides in a final volume of 25 μl containing the protein sample in water, 1.5×10^3 spores and 4% (w/v) sucrose. Controls were performed by replacing the protein sample with the same volume of water. After 16 h of incubation in the dark at 25 °C and at a 100% relative humidity, samples were microscopically evaluated for the presence of germinated spores.

Results

Distribution of Ha-AP10

To analyze the distribution of Ha-AP10 total proteins from seeds, hypocotyles, roots, leaves and flowers were fractionated by SDS-PAGE, and protein was detected by Western blot using an anti-Ha-AP10 antibody. Fig. 1A shows that Ha-AP10 was only specifically detected in total proteins from dried seeds (lane 3). The antibody also detected other proteins in extracts from leaves and flowers, but those signals can be considered nonspecific because they were also detected by the preimmune serum (Fig. 1B, lanes 1 and 2).

Evolution of Ha-AP10 levels during seed germination

To address the behavior of Ha-AP10 during germination, total proteins obtained from dried seeds and germinating seeds were fractionated by SDS-PAGE, and the levels of Ha-AP10 were analyzed by Western blot. As expected, the pattern of total

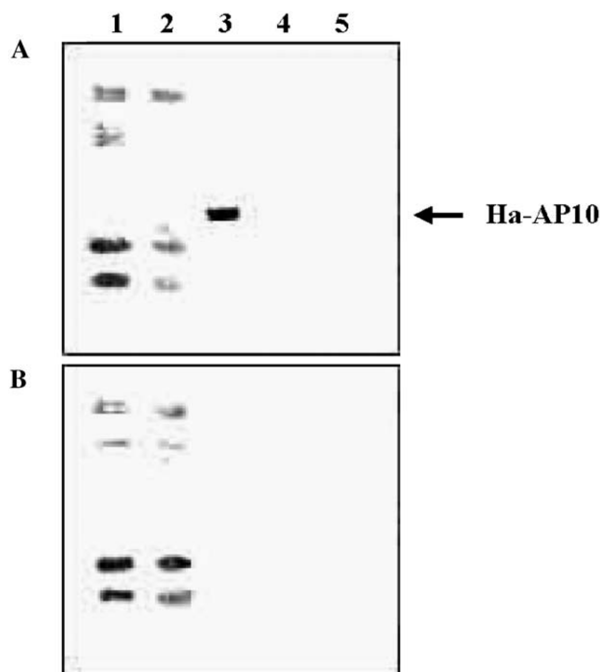

Figure 1. Distribution of Ha-AP10 in sunflower. Western blot of total protein extracts from leaves (lane 1), flowers (lane 2), dry seeds (lane 3), hypocotyls (lane 4) and radicles (lane 5), immunodetected with Ha-AP10 antibodies (panel A) or preimmune sera (panel B). Samples 1 and 2 were obtained from 3-month-old plants, whereas samples 4 and 5 were obtained from 12-day-old seedlings.

proteins detected with Coomassie blue is modified during germination, with some protein bands disappearing 5 days post-imbibition (Fig. 2A). However, Western blot analyses indicated that Ha-AP10 was still detected after 5 days of germination, and its levels tended to increase, although repetitive decay was observed on day 2 (Fig. 2B). Tissue printing experiments were then performed to gain insight into the localization of Ha-AP10 during seed germination. Fig. 3 shows that in dried seeds, Ha-AP10 was detected in all of the printed surface, which mainly corresponds to the cotyledons. In germinating seeds of 0, 1, 2 and 3 dpi, Ha-AP10 was also detected in the entire cotyledon. Although the localization of Ha-AP10 correlated with the pattern of total protein stained with Red Ponceau (compare Figs. 3B and A), the absence of signals detected in the presence of the preimmune serum (Fig. 3D) confirmed the specificity of the assay.

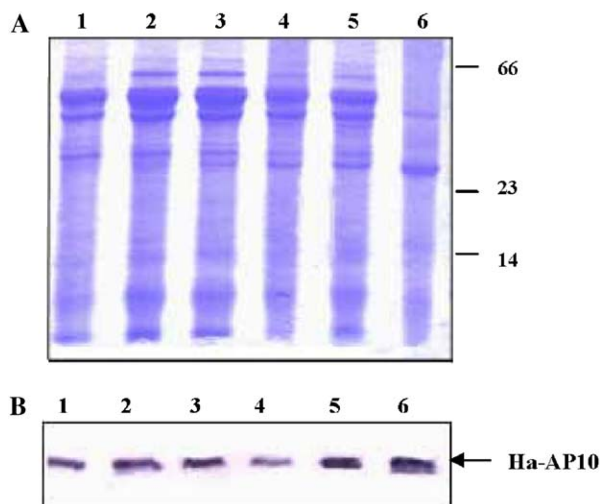

Figure 2. Analysis of the level of Ha-AP10 in germinating seeds of sunflower. (A) 30 μg of total proteins extracted from dry seeds (lane 1), and germinating seeds of 0 dpi (days post-imbibition) (lane 2), 1 dpi (lane 3), 2 dpi (lane 4), 3 dpi (lane 5) and 5 dpi (lane 6) were separated by SDS-PAGE and stained with Coomassie blue. Sizes of molecular markers (not shown) are indicated on the right in kilodaltons. (B) Immunoblotting with anti-Ha-AP10 antisera of the same samples detailed in (A).

Levels of Ha-AP10 under abiotic stress

Germinating seeds were treated with salt or exogenous application of ABA, and after 2 days of treatment the levels of Ha-AP10 were determined by Western blot. Fig. 4 revels that both 0.3 M NaCl and 5 μM ABA increased Ha-AP10 levels.

Levels of Ha-AP10 under biotic stress

Alternaria alternata is a fungal pathogen of sunflower which attacks the inflorescence and

Figure 4. Changes in the levels of Ha-AP10 in germinating sunflower seeds under abiotic stress. (A) Western blot of total proteins extracted from germinating seeds of 0 dpi treated during 2 days with water (control), ABA 5 μM or NaCl 300 mM.

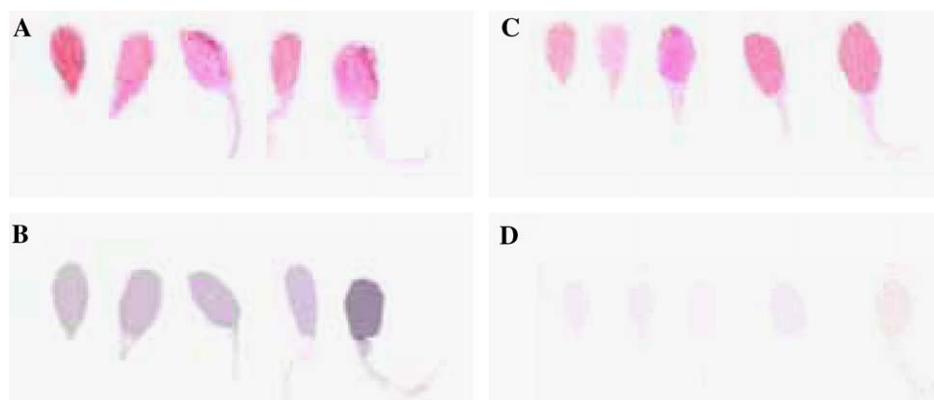

Figure 3. Tissue print immunolocalization of Ha-AP10 in cotyledons of germinating seeds. Dry seeds and germinating seeds of 0, 1, 2 and 3 dpi were cut into halves, and each half was blotted on nitrocellulose. (A, C) Sections stained with Red Ponceau for total protein. (B) Immunoblot incubated with anti-Ha-AP10 antisera. (D) Immunoblot incubated with pre-immune antisera.

infects the seeds, leading to a reduction in germination and degradation of seed components (Lagopodi and Thanassoupoulos, 1998). To analyze the effect of the fungus on the levels of Ha-AP10, sunflower seeds were inoculated with *A. alternata* mycelium, or were mock inoculated, and samples were obtained at 1, 2 and 3 days after treatment. Total proteins were then extracted and analyzed for the presence of Ha-AP10 by Western blot. Fig. 5 shows that the content of Ha-AP10 increased in germinating seeds upon infection.

Activity of Ha-AP10 against the pathogenic fungus *A. alternata*

The antifungal activity of Ha-AP10 has been previously described (Regente and de la Canal, 2000), but no data have been presented concerning the ability of the protein to inhibit the growth of a pathogen naturally attacking sunflower seeds. Fig. 6 shows the effect of different concentrations of Ha-AP10 on the germination of *A. alternata* fungal spores. It is evident that protein concentrations under 200 μg/ml were unable to produce a complete inhibition of spore germination, but a clear effect was detected at high protein doses.

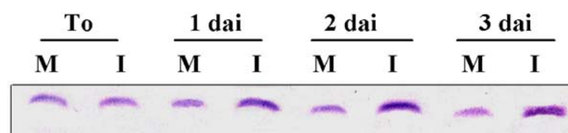

Figure 5. Changes in the levels of Ha-AP10 in germinating sunflower seeds inoculated with mycelium of the pathogenic fungus *Alternaria alternata*. (A) 30 μg of total proteins were extracted from inoculated germinating seeds (I) and mock inoculated germinating seeds (M). Samples were analyzed by Western blot at 0, 1, 2 and 3 days after inoculation (dai).

Discussion

LTPs constitute a large family of proteins whose different members seem to be implicated in diverse functions *in planta*. In this study we examined changes in Ha-AP10 levels in response to different stresses, and its evolution during seed germination as a first approach to understand its function. Upon examination of several plant parts, we detected the presence of Ha-AP10 only in seeds, which is consistent with the expression data previously obtained by Northern blot analysis (Regente and de la Canal, 2003). In addition, we observed that Ha-AP10 was present during the first days of germination and its levels tend to increase. However, the protein was no longer detected in 12-day-old seedlings, and no traces of the protein or its transcripts could be detected in mature leaves and flowers (Regente and de la Canal, 2003). LTP mRNAs are also present in germinating tomato and colza seeds (Torres-Schumann et al., 1992, Soufleri et al., 1996), and a similar pattern of expression has been described for two LTP-like genes and their protein products in *Euphorbia lagascae* (Edqvist and Farbos, 2002; Eklund and Edqvist, 2003), which have been shown to be present 5–6 days post-imbibition, reducing their levels thereafter.

We detected Ha-AP10 in the cotyledons of dry and germinating seeds, and the same location has been demonstrated for LTPs from castor bean, colza and *E. lagascae* (Tsuboi et al., 1992; Soufleri et al., 1996; Edqvist and Farbos, 2002; Eklund and Edqvist, 2003). Germination-specific LTPs are assumed to be involved in the mobilization of lipid reserves during germination (Tsuboi et al., 1992; Soufleri et al., 1996). This hypothesis is based on two main assumptions: first, that the expression of castor bean and colza seed LTPs coincide with the degradation of the oil bodies; and second, that LTPs are distributed on the entire cotyledon. In this sense, it must be taken into account that castor

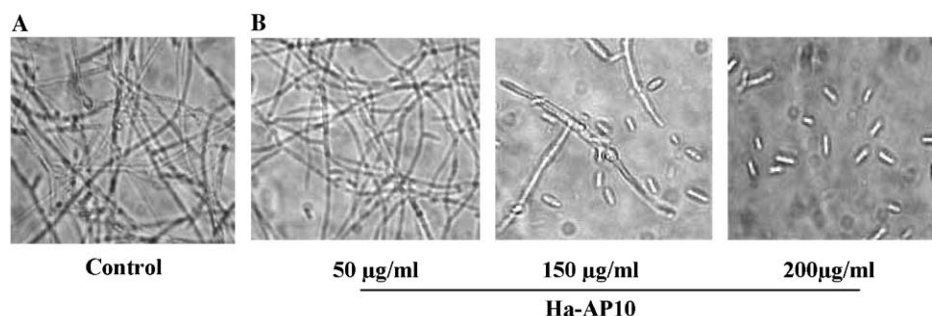

Figure 6. Antifungal activity of Ha-AP10 against the pathogenic fungus *Alternaria alternata*. Spores of *A. alternata* were incubated for 16 hs with water (control) (A) or with the indicated concentrations of Ha-AP10 (B). Afterwards, the morphology of the spores was analyzed by microscopic observation. Magnification 150 ×.

bean, colza, *E. lagascae* and sunflower are oily seeds, whose stored lipids are mobilized to support seedling growth. However, we must bear in mind that Ha-AP10 has been isolated based on interest in its antifungal properties. Antimicrobial peptides that participate in plant defense by killing invading microorganisms are mainly distributed on the surface of different plant parts or organs (Broekaert et al., 1997). This is also the case for many LTPs as, for example, those from carrot (Sterk et al., 1991), barley leaves (Molina and García Olmedo, 1993) and broccoli leaves (Pyee et al., 1994). On the other hand, tissue printing experiments demonstrate that Ha-AP10 is distributed on the entire cotyledon instead of concentrated in the epidermal tissues, as has been described for other antimicrobial Cys-rich peptides. Moreover, here we show that Ha-AP10 is able to inhibit the germination of spores of the phytopathogenic fungus *A. alternata*, but this effect was attained at high protein doses (200 μg/ml). Although the site-specific concentration of Ha-AP10 could not be determined, the protein concentration required for fungal inhibition in vitro was much greater than that frequently shown for antimicrobial peptides that participate in defense reactions (Broekaert et al., 1997). However, infected seeds showed increased levels of Ha-AP10. Because it is considered a pathogenesis related protein (Van Loon and Van Strein, 1999), this enhancement is not surprising, but according to the results discussed above it should be questioned whether the protein may participate in plant defense via a direct effect on fungi (as an antimicrobial protein), or by mediating defense responses by still unspecified pathways (Buhot et al., 2001; Maldonado et al., 2002). Similar results have been presented for LTP genes from grape, which are not able to reduce the growth of the fungus *Botrytis cinerea* in vitro, but their expression was induced in cell cultures treated with elicitors of the same pathogen (Gomès et al., 2003).

The induction of certain LTP genes upon abiotic stress is largely documented, and this induction is generally observed in plant tissues that do not display constitutive expression of those genes (Kader, 1996). Here we showed that in sunflower germinating seeds, the basal level of Ha-AP10 increased after salt and ABA treatment, suggesting that the protein may also participate in adaptative responses. A few reports have already shown an increase in LTP mRNA levels in germinating seeds upon salt stress or ABA treatment (Soufleri et al., 1996; Vignols et al., 1997). In this sense, our results constitute an interesting observation, because induction of LTP genes is frequently observed by Northern blot analysis, but protein levels are rarely analyzed. Therefore, it is not known whether these data really reflect the presence of the protein.

Although the data presented in this paper do not allow us to assign a specific function to Ha-AP10, it is tempting to speculate that Ha-AP10 may have a dual role in germinating seeds: the mobilization of lipid reserves, and adaptation to stress. However, the possibility that the induction of the protein after hormonal or stress treatment is just a consequence of enhanced energy requirements cannot be excluded.

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