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Unexpected localization of a lipid transfer protein in germinating sunflower seeds

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Summary

Plant lipid transfer proteins (LTPs) are low-molecular-mass proteins whose biological function still remains elusive. They are synthesized with a signal peptide that drives them to the secretory pathway. We have previously described the occurrence of an apoplastic LTP named Ha-AP10, present in sunflower seeds. Using a biochemical approach we now demonstrate that a fraction of Ha-AP10 is perispherically bound to membranes of germinating seeds. Purification of plasma membranes revealed the presence of Ha-AP10 in this fraction. Fluorimmunolocalization studies on germinating sunflower seeds demonstrated that in addition to the apoplastic and plasma membrane localization, Ha-AP10 is also present intracellularlly associated to unidentified structures. This varied distribution of Ha-AP10 in sunflower seeds may give novel clues to understand the role of LTPs in seed physiology.

Introduction

Lipid transfer proteins (LTPs) are low-molecular-mass proteins extensively studied in higher plants that are characterized by the presence of eight cysteine residues in conserved positions of their sequence. They have been classified into two families according to their molecular masses. Hence, LTP1 family is formed by 9–10 kDa peptides, while members of the LTP2 family present molecular masses around 7 kDa (Carvalho and Gomes, 2007; Kader, 1996).

The folding of LTPs, studied in mung bean (Lin et al., 2005), tomato (Rep et al., 2003) and several monocots (Douliez et al., 2001; Gomar et al., 1998; Hoh et al., 2005; Lee et al., 1998; Palacin et al.,

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Ha-AP10, *Helianthus annuus* antifungal protein 10 kDa; LTP, lipid transfer protein; PMSF, phenylmethyl sulfonilfluoride; SDS, sodium dodecyl sulfate.

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2007), revealed a similar global structure which includes a hydrophobic cavity that can accommodate a variety of lipids. In fact, the ability of some LTPs to bind and transfer lipid molecules *in vitro* has been demonstrated (Zachowski et al., 1998). Moreover, covalently linked lipid derivatives have been detected in LTPs (Bakan et al., 2006; Douliez et al., 2001).

Immunolocalization and biochemical approaches performed in several plant species revealed that most LTPs are localized in the apoplast (Thoma et al., 1993, 1994) or are secreted by cell cultures (Coutos-Thevenot et al., 1993; Pyee et al., 1994; Sterk et al., 1991). Although the great majority of plant LTPs are extracellular, two LTP1 have been reported intracellularly. They are localized in the glyoxysome matrix (Tsuboi et al., 1992) and in the protein storage vacuoles (Carvalho et al., 2004) of *Ricinus communis* and *Vigna unguiculata* seeds, respectively.

Several roles have been proposed for LTPs. To date, they have been implicated in wax and cutin assembly (Cameron et al., 2006; Hollenbach et al., 1997; Pyee and Kolattukudy, 1995; Sterk et al., 1991), antimicrobial defence through the inhibition of fungal and bacterial growth (Molina et al., 1993; Terras et al., 1992), pollen tube adhesion (Park et al., 2000), mobilization of seed storage lipids (Edqvist and Farbos, 2002; Tsuboi et al., 1992) and cell wall extension (Nieuwland et al., 2005). LTPs have also been associated with plant signalling. Hence, a member of the LTP2 family is involved in the generation or transmission of a molecular signal involved in the systemic acquired resistance in *Arabidopsis* (Maldonado et al., 2002) and two LTPs are capable to bind *in vitro* to a plasma membrane receptor involved in defence responses (Buhot et al., 2001, 2004). This vast and rather confusing panorama may be originated by the fact that LTPs are encoded by multigenic families whose members display low sequence identity and putatively accomplish distinct function.

We have previously characterized a LTP from sunflower (*Helianthus annuus*) seeds, Ha-AP10, that displays antifungal activity (Regente and de la Canal, 2000). Ha-AP10 expression persists during germination and is induced upon salt stress, fungal infection and ABA treatment (Gonorazky et al., 2005). Since Ha-AP10 has been detected in extracellular fluids of sunflower seeds and its cDNA clone has a secretory signal peptide sequence, the targeting of the protein to the apoplastic space has been assumed (Regente and de la Canal, 2003). Although this location is the typical one for plant LTPs, preliminary data obtained in our laboratory suggested that a fraction of Ha-AP10 may be bound to microsomes. Therefore, the aim of this work was to investigate the subcellular localization of Ha-AP10 during germination and contribute in the interpretation of its role in sunflower seeds, on the assumption that the subcellular localization of a protein has an intimate connection with its biological function (Ephritikhine et al., 2004).

Materials and methods

Plant material and microsomes preparation

Sunflower (*Helianthus annuus* L.) seeds, line 10347, were kindly supplied by Advanta Semillas SAIC, Argentina. After 24 h of water imbibition at 25 °C, seeds were decorticated and homogenized. The extraction buffer contained 50 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 2 mM EDTA, 5 μg/mL PMSF. The insoluble debris were removed by centrifugation at 600*g* for 15 min. Microsomal membranes and membrane-free supernatant were then isolated according to Hawes and Satiat-Jeunemaitre (2001). Briefly, the homogenate was submitted to centrifugation at 25.000*g* for 20 min at 4 °C, the supernatant was removed (soluble fraction) and the pellet was then dissolved in five volumes of extraction buffer and homogenized by using a plastic pestle. The homogenate was centrifuged again at 25.000*g* for 20 min to obtain the final microsomal fraction which was suspended in five volumes of extraction buffer.

Glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate (Glu-6-P) dehydrogenase activity (EC 1.1.1.49) was measured at 25 °C in a solution containing 65 mM $MgCl_2$, 1.3 mM $NADP^+$, 85 mM triethanolamine buffer pH 7.6 and 20–160 μg of microsomal or soluble protein. The reaction was initiated by the addition of 3 mM Glu-6-P. Glu-6-P dehydrogenase activity was determined by following the appearance of NADPH at 340 nm (Pinedo et al., 1993).

Protein analyses

Protein determinations were performed by the bicinchoninic acid protein assay using bovine serum albumin (BSA) as standard. SDS–PAGE was performed according to Laemmli (1970) in 12% acrylamide gels and the sample buffer contained 50 mM Tris pH 6.8, 2% SDS and 10% glycerol. After electrophoretic separation, proteins were electroblotted onto a nitrocellulose membrane in 39 mM

glycine, 0.0376% SDS, 48 mM Tris and 20% methanol (final pH: 9.2). Membranes were then incubated with blocking buffer (100 mM Tris–HCl pH 8, 1% BSA) for 30 min. A mouse antiserum raised against Ha-AP10 was used diluted 1:6000 in blocking buffer as described in Regente and de la Canal (2000). Blots were washed and then incubated for 2 h with horseradish peroxidase-conjugated anti-mouse IgG antibodies. Signal detection was performed as described elsewhere (Regente and de la Canal, 2000).

In sílico analysis

Ha-AP10 amino-acid sequence deduced from its cDNA clone (Regente and de la Canal, 2003) was analyzed for the presence of specific domains using the following programs: "DAS" (http://www.sbc.su.se/nmiklos/DAS) and "Multiple TMAP" (http://www.mbb.ki.se/tmap) for transmembrane domains; "SOSUI" (http://www.sosui.proteome.bio.tuat.ac.jp) and "TMHMM 2.0" (http://www.cbs.dtu.dk/services/TMHMM) for alpha helixes; "TMBETA-SVM" (http://tmbeta-smv.cbrc.jp) and "TMB-Hunt" (http://www.bioinformatics-.leeds.ac.uk) for beta barrels; "Myristoylator" (http://www.expasy.ch/tools/myristoylator) and "NMT" (http://mendel.imp.univie.ac.at) for myristoylation; "Pre-Ps" (http://mendel.imp.univie.ac.at) for farnesylation; "Big PI Predictor" (http://mendel.imp.univie.ac.at) and "dGPI" (http://129.194.185.165/dgpi/dgpi) for glycosylphosphatidylinositol-anchoring; "Wolf PSORT" (http://psort.ims.u-tokyo.ac.jp) and "Target P 1.1" (http://www.cbs.dtu.dk/services/TargetP) for subcellular localization.

Analysis of Ha-AP10 membrane association

Equal amounts of total microsomes were incubated for 1 h with the following agents: 50 mM Tris–HCl buffer pH 7.5 (control), 0.1 M Na_2CO_3 (pH 11), 0.6 M NaCl, 4% Triton X-100 or 2% SDS. Incubations were performed on ice or 25 °C (SDS sample) and then centrifuged for 20 min at 25.000*g* to separate the insoluble membrane materials in the pellet from the solubilized proteins in the supernatant (Harkins et al., 2001). These fractions were then analyzed by SDS-PAGE and immunoblotting. Purification of peripheral proteins was performed as described in Bordier (1981). Briefly, microsomal membranes were suspended in 100 mM potassium phosphate buffer, pH 7.4, and partitioned three times in 2.4% precondensed Triton X-114. This was done by incubating the Triton X-114/membrane mixture for 15 min on ice, removing insoluble materials by centrifugation at 10.000*g* at 4 °C for 10 min, heating the mixture at 37 °C for 5 min, and then separating the phases by spinning at 1000*g* at room temperature for 10 min. The aqueous upper phase containing peripherally associated proteins was analyzed for the presence of Ha-AP10 by protein gel blot.

Isolation of plasma membrane

Plasma membrane vesicles were purified from the microsomal fraction of imbibed seeds using the two-phase partitioning procedure as described elsewhere (Larsson et al., 1987). The optimal conditions for separation were determined by systematic variation of the polymer concentration and salt composition. The phase system containing 6.5% (w/w) dextran T-500, 6.5% (w/w) polyethylene glycol 3350, 0.33 M sucrose, 5 mM potassium phosphate pH 7.8 and 5 mM KCl proved to be the best. Final pellets were suspended in a medium containing 0.33 M sucrose, 5 mM potassium phosphate pH 7.8, 5 mM KCl and 0.1 mM EDTA for protein gel blot analysis or in 50 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 2 mM EDTA, 5 μg/mL PMSF for ATPase activity assays (EC. 3.6.1.35). Plasma membrane fraction enrichment was estimated by comparing the plasma membrane ATPase activity with reference to other endomembrane ATPase activities according to Lanzetta et al. (1979) with modifications. ATPase activity was determined by measuring inorganic phosphate release at 30 °C in 5 mM Tris–MES (morpholineethanesulfonic acid) buffer pH 6.5 (for plasma membrane and tonoplast ATPase activity) or 5 mM Tris–HCl buffer pH 8 (for mitochondrial ATPase activity) containing 3 mM $MgCl_2$, 3 mM ATP, 55 mM KCl and 1 mM sodium molybdate. The reaction (total volume, 250 μL) was stopped after 20, 30 and 40 min by immediately adding 750 μL of a malachite green solution (0.034% (w/w) malachite green, 1.05% (w/w) ammonium molybdate in 1 M HCl) and 100 μL of a 34% (w/w) sodium citrate solution. The absorbance at 660 nm was immediately measured. One unit of ATPase activity was defined as the amount of enzyme that released 1 μmol of inorganic phosphate in 1 min. To distinguish between the ATPase activities of different compartments, protein extracts were also incubated with 0.1 mM *ortho*-vanadate (plasma membrane ATPase inhibitor), 10 mM nitrate (K_2NO_3, mitochondrial ATPase inhibitor) or 0.1 mM azide (NaN_3, tonoplast ATPase inhibitor). Enrichment of the plasma membrane fraction was also confirmed by protein gel blot analysis using a plasma

membrane ATPase antibody that recognizes sunflower ATPase specifically (Parets-Soler et al., 1990).

Paraplast® sections

Sunflower seeds were imbibed in water for 24 h, decorticated, sectioned in 2 mm width slices and embedded in 10% dimethyl sulphoxide (DMSO). Then the slices were subjected to vacuum until boiling of the solution was reached (three times). The DMSO solution was then replaced for fixative solution (0.05 M phosphate pH 7.4, 10% DMSO, 4% paraphormaldehyde, 1% glutaraldehyde and 0.06 M sucrose) and incubated at 4 °C for 3–4 h. The slices were then dehydrated by running them through an ethanol–xylene series as follows: 30% ethanol, 2 × 30 min; 50% ethanol, 2 × 30 min; 70% ethanol, 2 × 30 min; 96% ethanol, 2 × 24 h at 4 °C; 3:1 ethanol: xylene, 2 h; 1:1 ethanol: xylene, 2 h; 1:3 ethanol: xylene, 2 h; xylene, 1 h; xylene overnight; xylene 1 h. When the samples were thoroughly infiltrated with xylene, they were placed in a 65 °C incubator and run through xylene-melted Paraplast series as follows: 3:1 xylene: Paraplast®, 2 h; 1:1 xylene: Paraplast®, 2 h; 1:3 xylene: Paraplast®, 2 h; Paraplast®, 1 h (twice); Paraplast®, overnight. Finally, the samples were poured into wood molds and the Paraplast was allowed to solidify. Sections (6, 7 or 10 μm) were cut from these blocks using a rotary microtome. The Paraplast® sections were collected on albumin-coated slides and dried for 24 h.

Fluorimmunolocalization and confocal microscopy

Seed sections were first deparaplasted and rehydrated through ethanol series as follows: 12 h xylene, 1 h fresh xylene, 100% ethanol, 5 min; 96% ethanol, 5 min; 90% ethanol, 5 min; 80% ethanol, 5 min; 70% ethanol, 5 min; 50% ethanol, 5 min; 30% ethanol, 5 min; distilled water, 5 min; PBS, 5 min. Labeling with Ha-AP10 antiserum (1:1000) and Alexa Fluor 488-conjugated goat anti-mouse IgG as secondary antibody (1:500) was carried out according to Gillespie (2005). Images were collected on an Olympus FV300 confocal laser-scanning microscope with argon laser excitation at 488 nm (Universidad de Buenos Aires, Argentina).

Results

Protein gel blot analyses were performed on soluble (cytosol and extracellular fluids) and microsomal protein extracts of 24 h imbibed sunflower seeds. The microsomes were prepared in a non-isotonic medium that produces organelle disruption and hence contain most membrane systems of the cell including the plasma membranes. As seen in Figure 1, and in agreement with the extracellular localization of Ha-AP10 (Regente and de la Canal, 2000), antibodies raised against this protein recognized a band of 10 kDa in soluble protein fractions. Besides, Ha-AP10 was also detected in microsomal fractions (Figure 1A). The signals detected by the Ha-AP10 antibody proved to be specific, since no protein was detected using preimmune serum (Figure 1B). Figure 1C shows that the protein patterns of soluble and microsomal fractions are clearly distinct, suggesting that the membrane fraction prepared is not significantly contaminated with soluble proteins. The same conclusion was obtained on the basis of the activity of the cytosolic marker glucose-6-phosphate dehydrogenase indicating that microsomes contained barely detectable levels of activity (Table 1).

As a first approach to unravel how Ha-AP10 is bound to microsomes, we performed *in silico* analyses. Ha-AP10 amino acid sequence deduced from its cDNA clone (Regente and de la Canal, 2003) was examined for the presence of transmembrane domains and consensus sequences for post-translational modifications that could anchor Ha-AP10 to membranes (described in Materials and methods). These analyses not only failed to detect any evidence of putative association of Ha-AP10 to membranes but also predicted an extracellular fate.

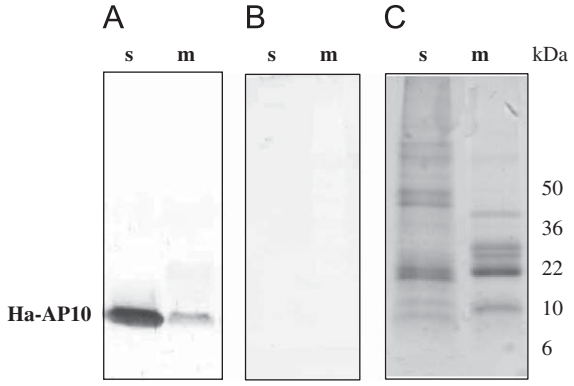

Figure 1. Ha-AP10 is present in both soluble and microsomal fractions of sunflower seeds. Soluble (s) and microsomal (m) proteins (100 μg) obtained as described in "Materials and methods" were analyzed by SDS-PAGE and immunoblotting using anti-Ha-AP10 (A) or preimmune serum (B). Protein profiles of both fractions were analyzed by SDS-PAGE and Coomasie blue staining (C). Molecular weight markers are indicated on the right.

An experimental approach was then set up to assess if Ha-AP10 was associated to microsomal membranes as a peripheral or integral protein. Aliquots of this fraction were treated with different agents that disrupt either electrostatic (0.6 M NaCl or 0.1 M Na_2CO_3) or hydrophobic (4% Triton X-100) interactions. Control treatments were also performed incubating the microsomes with buffer alone or in the presence of the ionic detergent SDS (2%) that leads to a complete disruption of the bilayer (positive control). After treatment, each sample was centrifuged to recover the soluble proteins released by the treatment and the remaining microsomal pellet. Protein gel blot analysis using Ha-AP10 antiserum showed that all the treatments were able to release the protein from the microsomal fraction to the supernatant, at least partially, while control buffer did not modify this association (Figure 2A). Even though the treatments were not exhaustive and a fraction of Ha-AP10 remained associated to the microsomes, these results indicate that Ha-AP10 is peripherally bound to membranes by a contribution of both electrostatic as well as hydrophobic interactions. This presumption was confirmed by applying a protocol specifically designed for the isolation of peripheral membrane proteins, based on the ability of Triton X-114 to interact with hydrophobic proteins and generate micellar solutions (Bordier, 1981). The presence of Ha-AP10 in the isolated peripheral protein fraction was revealed by immunodetection (Figure 2B). All together, these experiments demonstrate that a fraction of Ha-AP10 is peripherally bound to membranes.

Table 1. Evaluation of cytosolic contamination in microsomes.

Fraction	Glucose 6-P dehydrogenase activity [U (min g prot)$^{-1}$]
Soluble	26.38 ± 3.75 (99.50)
Microsomes	0.10 ± 0.04 (0.50)
Total	26.49 ± 3.80 (100.00)

Soluble and microsomal glucose-6-phosphate dehydrogenase activity, one activity unit (U) was defined as the amount of enzyme that produces a change of 1 in the absorbance. The results are expressed as U (min g prot)$^{-1}$. Numbers in parentheses represent percentage of activity relative to the "Total" value. Each value represents means ± sd of three replicate experiments. Each replicate experiment was performed using independent membrane preparations.

In order to determine the specific localization of membrane-bound Ha-AP10, we first looked for its presence in the plasmalema, on the basis of its proximity to the apoplast and the results reported by Buhot et al. (2001) on the binding of a LTP to a plasma membrane receptor. Plasma membranes were purified from microsomes by two phase partitioning according to Larsson et al. (1987). To assay the enrichment in plasma membranes of the fractions obtained, they were tested for the activity of the vanadate-sensitive ATPase marker (Table 2). The activity of tonoplast and mitochondrial enzyme markers (nitrate-sensitive and azide-sensitive ATPases, respectively) showed that the plasma membrane preparation was essentially free of contamination by other membrane fractions (Table 2). So, this plasma membrane enriched sample was further analyzed by protein gel blot to assess the presence of Ha-AP10. As shown in Figure 3, Ha-AP10 antiserum detected the protein

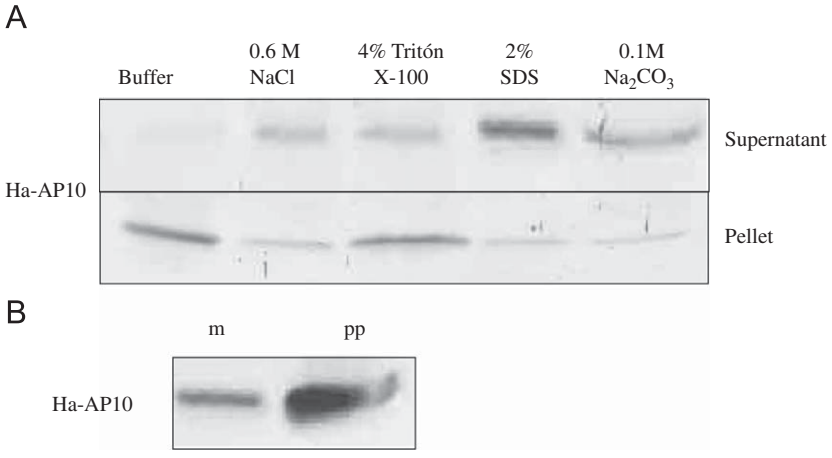

Figure 2. Peripheral association of Ha-AP10 to microsomes. Immunoblotting using anti-Ha-AP10 serum. (A) Microsomes treated with buffer, 0.6 M NaCl, 4% Triton X-100, 2% SDS or 0.1 M Na_2CO_3 were centrifuged to separate proteins released by the treatment (supernatant) from proteins that remain associated to membranes (pellet). (B) Microsomal (m) and peripheral proteins (pp).

Table 2. Determination of plasma membrane enrichment.

Marker enzyme	Microsomes	Plasma membrane
Vanadate-sensitive ATPase (plasma membrane)	9.24±2.95 (53.18)	38.01±13.53 (93.49)
Nitrate-sensitive ATPase (tonoplast)	3.01±3.96 (14.45)	2.22±3.84 (6.51)
Azide-sensitive ATPase (mitochondria)	5.41±1.29 (32.66)	0.00±0.00 (0.00)
Total	17.61±5.40 (100)	40.23±11.29 (100)

ATPase activity was assayed in microsomes or plasma membranes fractions, as described in ''materials and methods''. Activities are expressed as nmol PO_4 mg prot^{-1} min^{-1}. Numbers in parentheses represent percentage of activity relative to the total value. Data represent means±sd of three replicate experiments. Each replicate experiment was performed using independent membrane preparations.

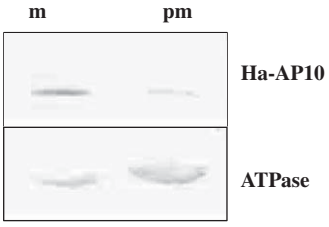

Figure 3. Ha-AP10 is present in plasma membrane preparations. Plasma membrane vesicles were analyzed by SDS-PAGE and immunoblotting using Ha-AP10 antiserum (upper panel) and anti-plasma membrane H^+-ATPase (lower panel). (m) Microsomal membranes; (pm) plasma membrane.

in plasma membrane fractions. A positive control with an H^+-ATPase antiserum confirmed the quality of the plasma membrane fraction obtained. Several repetitions of this experiment showed that only a low portion of Ha-AP10 observed in microsomes was further detected in the plasma membrane fraction, suggesting that Ha-AP10 may be also bound to other microsomal membranes. To further investigate additional localizations of Ha-AP10, we performed immunofluorescence experiments on paraffin-embedded longitudinal sections of sunflower germinating seeds. Concerning its tissue distribution, confocal microscopic observations revealed that Ha-AP10 is detected in the entire surface of the cotyledons and in the shoot meristem, as well as in the inter-cotyledon space (Figure 4A). In addition, Ha-AP10 is particularly concentrated in primary xylem and immature vascular elements (Figure 4C–F). A striking observation in these images is that, besides its extracellular location, we observed Ha-AP10 presence in intracellular compartments (Figure 4). A close examination of Ha-AP10's cellular localization confirms its binding to plasma membranes and also reveals the presence of the protein associated with intracellular bodies (Figure 5A). Labeling with preimmune serum (Figure 5C) or secondary antibody alone (Figure 5E) does not show any fluorescence, indicating that the signal observed with Ha-AP10 antibodies is specific. Due to the extensive compacting of sunflower seed cells, care must be taken in determining the identity of the intracellular compartments to which Ha-AP10 is associated. Preliminary observations using labeled markers made us difficult to unambiguously assign a subcellular localization to Ha-AP10 in sunflower seeds. Hence, Ha-AP10 intracellular presence was confirmed but its precise location still remains under study.

Discussion

The LTPs cloned so far contain a signal peptide responsible for their insertion in the endoplasmic reticulum and subsequent secretion of the protein. Hence, the classical interpretations of the role of LTPs in seeds take account of their extracellular location and, according to their pattern of expression and biological activities, their participation in defence reactions towards pathogen attack has been proposed (Canevascini et al., 1996; Regente and de la Canal, 2000; Terras et al., 1992; Wang et al., 2004; Wijaya et al., 2000; Yang et al., 2006). However, a few LTPs have also been suggested to participate in the mobilization of storage lipids (Edqvist and Farbos, 2002; Tsuboi et al., 1992). We now demonstrate that Ha-AP10, a LTP1 enriched in extracellular fluids of sunflower seeds, is present in intracellular organelles and also in the plasma membrane. This is not the typical location described for LTPs and it was not expected for Ha-AP10 previously detected in the apoplast and whose cDNA presents a signal peptide. However, another LTP1 from *Vigna unguiculata* seeds has been shown to present both extracellular and intracellular localization. It was demonstrated that this LTP is localized in the lumen of vesicles derived from protein storage vacuoles as well as in small bodies suggested to be lipid containing vesicles (Carvalho et al., 2004). This evidence reveals that the case of Ha-AP10 is not an exception and this

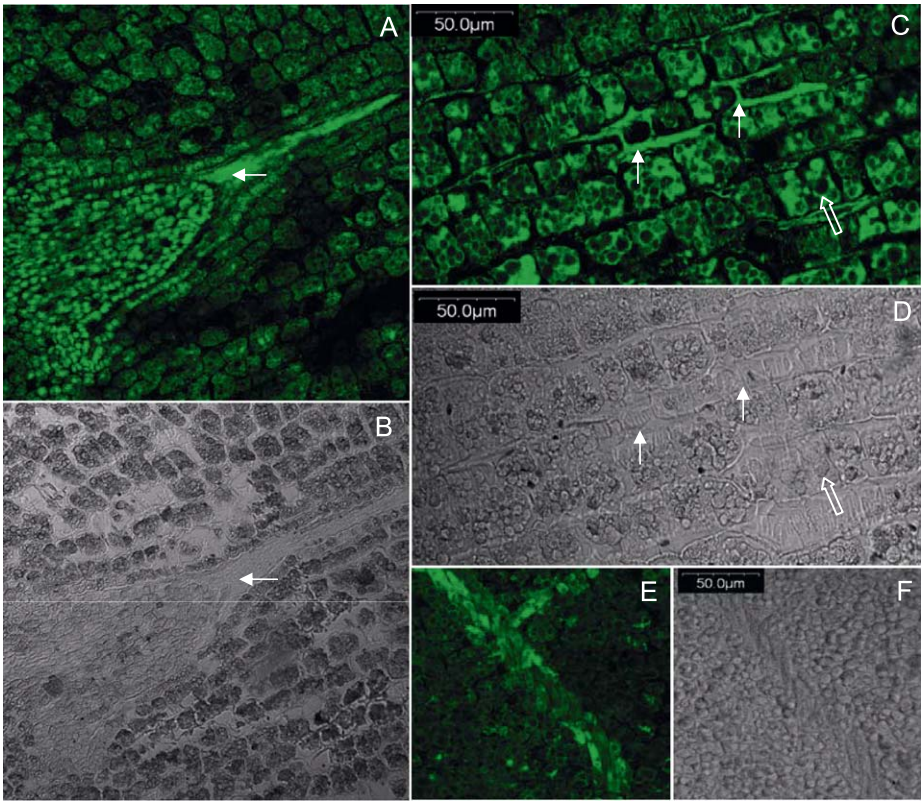

Figure 4. Immunolocalization of Ha-AP10. (A) Confocal laser scanning microscopy showing immunofluorescence labeling (green) of Ha-AP10 in cotyledons and inter-cotyledon space. (C) Confocal microscopy showing Ha-AP10 localization in primary xylem and intercellular space. (E) Confocal microscopy showing Ha-AP10 localization in immature vascular elements. (B, D and F) Bright field transmission microscopy of sections A, C and E respectively. Solid and open arrows point the extracellular and intracellular localization of Ha-AP10 respectively. Bar scale: 20 µm.

double localization (intra and extracellular) could be part of a complex and still unknown process. Interestingly, to our knowledge the intracellular LTPs reported so far have all been detected in seeds and belong to the same family (LTP1), reinforcing the idea that they may have a specific function in seed maturation and/or germination. In fact, we have previously demonstrated that total levels of Ha-AP10 remain unchanged during imbibition and tissue printing experiments showed no evident modification of its distribution (Gonorazky et al., 2005), but this evidence does not preclude a subcellular relocalization of the protein during imbibition.

As LTPs constitute multigenic families, a putative explanation of our results could be the existence of different gene products with high identity that could be recognized in diverse compartments by the Ha-AP10 antiserum. However, a screening of cDNA clones from sunflower seeds performed in our laboratory only detected the sequence of Ha-AP10 (Regente and de la Canal, 2000), suggesting that no other similar LTPs are expressed in sunflower seeds. In addition, exploration of the sunflower database (TIGR gene index, release June 19, 2008) revealed the existence of some ESTs similar to Ha-AP10, but their identity is below 70% and their expression in seeds is uncertain.

In this article we provide evidence demonstrating that a fraction of the extracellular LTP Ha-AP10 is bound to plasma membranes in germinating seeds. The presence of Ha-AP10 in the plasma membrane could be consistent with a previous observation of Buhot et al. (2001), who demonstrated that a LTP from wheat endosperm was able to bind to elicitin receptors from tobacco plasma membranes *in vitro*. Experiments conducted using a recombinant form of a tobacco LTP also proved that the protein binds specifically to membrane fractions *in vitro* (Buhot et al., 2004). Taken together these evidences have prompted the authors to suggest a role of LTPs in plant defence signalling. Other apoplastic proteins have been shown to interact with plasma membrane through specific receptors. A well known example is the interaction of tomato Cf receptor in plasma membrane with apoplastic avirulence peptides secreted by the fungus *Cladosporium fulvum* (Rivas and Thomas,

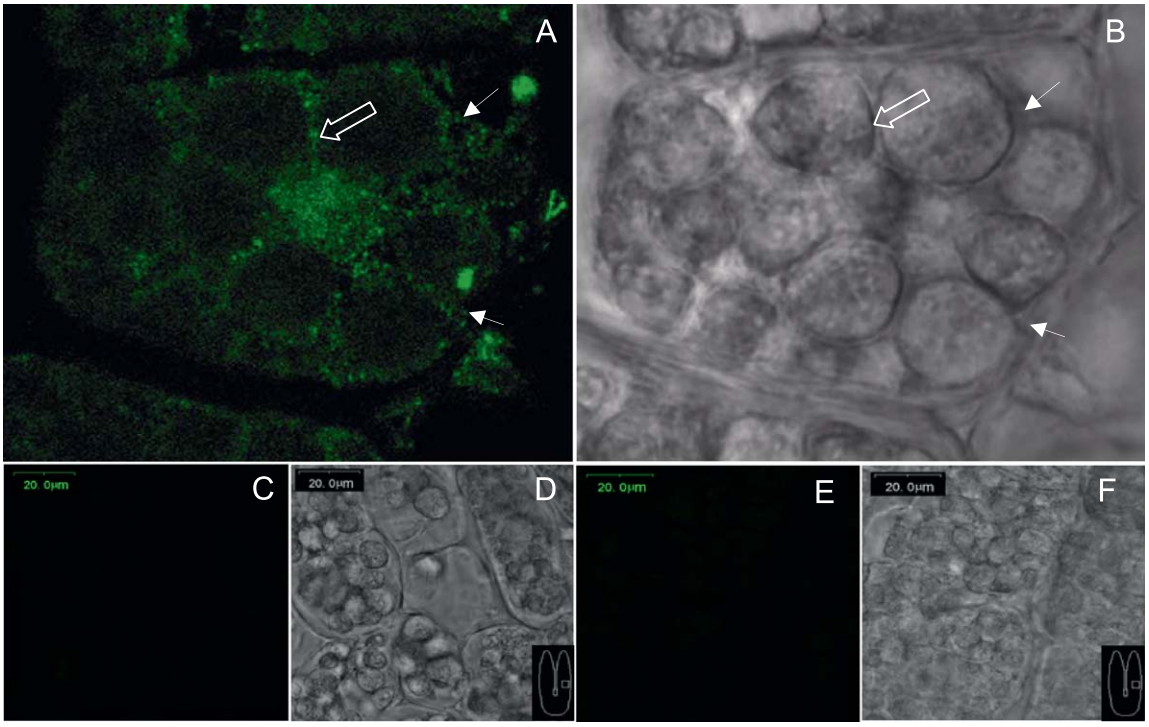

Figure 5. Intracellular localization of Ha-AP10. Confocal laser scanning microscopy showing immunofluorescence labeling of Ha-AP10 (A); preimmune serum (C); or Alexa Fluor 488-conjugated goat anti-mouse IgG antibody alone (E). B, D and F: bright field transmission microscopy of sections A, C and E respectively. Solid and open arrows point the plasma membrane and intracellular bodies' localization of Ha-AP10 respectively. Bar scale: 20 μm.

2005). Another case is Clavata 3 protein which acts as an extracellular signalling molecule that requires the interaction with the receptor complex CLV1/CLV2 to exert its function in shoot apical meristem to allow plant growth and development (Fiers et al., 2007; Rojo et al., 2002). According to the evidence discussed, the presence of Ha-AP10 associated to plasma membranes could account for a role in signalling events during seed germination accomplished by interaction with a putative plasma membrane receptor. However, other interpretations are also plausible taking into account the data provided in this work. The fact that we have also detected Ha-AP10 in intracellular bodies as well as in plasma membranes could suggest that membrane traffic events may be occurring during seed imbibition. Unfortunately sunflower seeds are so tightly packed that our efforts to undoubtedly identify Ha-AP10 intracellular localization were unsuccessful. Work is in progress to elucidate the biological significance of this finding.

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References

Bakan B, Hamberg M, Perrocheau L, Maume D, Rogniaux H, Tranquet O, et al. Specific adduction of plant lipid transfer protein by an allene oxide generated by 9-lipoxygenase and allene oxide synthase. J Biol Chem 2006;281:38981–8.

Bordier C. Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem 1981; 256:1604–7.

Buhot N, Douliez JP, Jacquemard A, Marion D, Tran V, Maume BF, et al. A lipid transfer protein binds to a

receptor involved in the control of plant defence responses. FEBS Lett 2001;509:27–30.

Buhot N, Gomes E, Milat ML, Ponchet M, Marion D, Lequeu J, et al. Modulation of the biological activity of a tobacco LTP1 by lipid complexation. Mol Biol Cell 2004;15:5047–52.

Cameron KD, Teece MA, Smart LB. Increased accumulation of cuticular wax and expression of lipid transfer protein in response to periodic drying events in leaves of tree tobacco. Plant Physiol 2006;140:176–83.

Canevascini S, Caderas D, Mandel T, Fleming AJ, Dupuis I, Kuhlemeier C. Tissue-specific expression and promoter analysis of the tobacco ltp1 gene. Plant Physiol 1996;112:513–24.

Carvalho AO, Gomes VM. Role of plant lipid transfer proteins in plant cell physiology—a concise review. Peptides 2007;28:1144–53.

Carvalho AO, Teodoro CES, Da Cunha M, Okorokova-Facanha AL, Okorokov LA, Fernandes KVS, et al. Intracellular localization of a lipid transfer protein in Vigna unguiculata seeds. Physiol Plant 2004;122: 328–36.

Coutos-Thevenot P, Jouenne T, Maes O, Guerbette F, Grosbois M, Le Caer JP, et al. Four 9-kDa proteins excreted by somatic embryos of grapevine are isoforms of lipid-transfer proteins. Eur J Biochem 1993;217:885–9.

Douliez JP, Jegou S, Pato C, Larre C, Molle D, Marion D. Identification of a new form of lipid transfer protein (LTP1) in wheat seeds. J Agric Food Chem 2001;49: 1805–8.

Edqvist J, Farbos I. Characterization of germination-specific lipid transfer proteins from *Euphorbia lagascae*. Planta 2002;215:41–50.

Ephritikhine G, Ferro M, Rolland N. Plant membrane proteomics. Plant Physiol Biochem 2004;42:943–62.

Fiers M, Ku KL, Liu C-M. CLE peptide ligands and their roles in establishing meristems. Curr Opin Plant Biol 2007;10:39–43.

Gomar J, Sodano P, Sy D, Shin DH, Lee JY, Suh SW, et al. Comparison of solution and crystal structures of maize nonspecific lipid transfer protein: a model for a potential in vivo lipid carrier protein. Proteins 1998;31:160–71.

Gonorazky AG, Regente MC, de la Canal L. Stress induction and antimicrobial properties of a lipid transfer protein in germinating sunflower seeds. J Plant Physiol 2005;162:618–24.

Harkins HA, Page N, Schenkman LR, De Virgilio C, Shaw S, Bussey H, et al. Bud8p and Bud9p, proteins that may mark the sites for bipolar budding in yeast. Mol Biol Cell 2001;12:2497–518.

Hawes C, Satiat-Jeunemaitre B. Plant Cell Biology: a practical approach. New York: Oxford University Press; 2001.

Hoh F, Pons JL, Gautier MF, de Lamotte F, Dumas C. Structure of a liganded type 2 non-specific lipid-transfer protein from wheat and the molecular basis of lipid binding. Acta Crystallogr D Biol Crystallogr 2005;61:397–406.

Hollenbach B, Schreiber L, Hartung W, Dietz KJ. Cadmium leads to stimulated expression of the lipid transfer protein genes in barley: implications for the involvement of lipid transfer proteins in wax assembly. Planta 1997;203:9–19.

Kader JC. Lipid-transfer proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 1996;47:627–54.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA. An improved assay for nanomole amounts of inorganic phosphate. Anal Biochem 1979;100:95–7.

Larsson C, Widell S, Kjellbom P. Preparation of high-purity plasma membranes. Methods Enzymol 1987; 148:558–68.

Lee JY, Min K, Cha H, Shin DH, Hwang KY, Suh SW. Rice non-specific lipid transfer protein: the 1.6 a crystal structure in the unliganded state reveals a small hydrophobic cavity. J Mol Biol 1998;276:437–48.

Lin KF, Liu YN, Hsu ST, Samuel D, Cheng CS, Bonvin AM, et al. Characterization and structural analyses of nonspecific lipid transfer protein 1 from mung bean. Biochemistry 2005;44:5703–12.

Maldonado AM, Doerner P, Dixon RA, Lamb CJ, Cameron RK. A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. Nature 2002;419:399–403.

Molina A, Ahl Goy P, Sánchez-Monge P, Garcia-Olmedo F. Inhibition of bacterial and fungal plant pathogens by thionins of types I and II. Plant Sci 1993;92: 169–77.

Nieuwland J, Feron R, Huisman BA, Fasolino A, Hilbers CW, Derksen J, et al. Lipid transfer proteins enhance cell wall extension in tobacco. Plant Cell 2005;17: 2009–19.

Palacin A, Quirce S, Armentia A, Fernandez-Nieto M, Pacios LF, Asensio T, et al. Wheat lipid transfer protein is a major allergen associated with baker's asthma. J Allergy Clin Immunol 2007;120:1132–8.

Parets-Soler A, Pardo JM, Serrano R. Immunocytolocalization of Plasma Membrane H-ATPase. Plant Physiol 1990;93:1654–8.

Park SY, Jauh GY, Mollet JC, Eckard KJ, Nothnagel EA, Walling LL, et al. A lipid transfer-like protein is necessary for lily pollen tube adhesion to an in vitro stylar matrix. Plant Cell 2000;12:151–64.

Pinedo ML, Segarra C, Conde RD. Occurrence of two endoproteinases in wheat leaf intercellular washing fluid. Physiol Plant 1993;88:287–93.

Pyee J, Kolattukudy PE. The gene for the major cuticular wax-associated protein and three homologous genes from broccoli (*Brassica oleracea*) and their expression patterns. Plant J 1995;7:49–59.

Pyee J, Yu H, Kolattukudy PE. Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. Arch Biochem Biophys 1994;311:460–8.

Regente MC, de la Canal L. Purification, characterization and antifungal properties of a lipid-transfer protein

from sunflower (*Helianthus annuus*) seeds. Physiol Plant 2000;110:158–63.

Regente M, de la Canal L. A cDNA encoding a putative lipid transfer protein expressed in sunflower seeds. J Plant Physiol 2003;160:201–3.

Rep M, Dekker HL, Vossen JH, de Boer AD, Houterman PM, de Koster CG, et al. A tomato xylem sap protein represents a new family of small cysteine-rich proteins with structural ilarity to lipid transfer proteins. FEBS Lett 2003;534:82–6.

Rivas S, Thomas CM. Molecular interactions between tomato and the leaf mold pathogen *Cladosporium fulvum*. Annu Rev Phytopathol 2005;43:395–436.

Rojo E, Sharma VK, Kovaleva V, Raikhel NV, Fletcher JC. CLV3 Is Localized to the Extracellular Space, Where It Activates the Arabidopsis CLAVATA Stem Cell Signaling Pathway. Plant Cell 2002;14:969–77.

Sterk P, Booij H, Schellekens GA, Van Kammen A, De Vries SC. Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 1991;3:907–21.

Terras FR, Goderis IJ, Van Leuven F, Vanderleyden J, Cammue BP, Broekaert WF. In vitro antifungal activity of a radish (*Raphanus sativus* L.) seed protein homologous to nonspecific lipid transfer proteins. Plant Physiol 1992;100:1055–8.

Thoma S, Kaneko Y, Somerville C. A non-specific lipid transfer protein from Arabidopsis is a cell wall protein. Plant J 1993;3:427–36.

Thoma S, Hecht U, Kippers A, Botella J, De Vries S, Somerville C. Tissue-specific expression of a gene encoding a cell wall-localized lipid transfer protein from Arabidopsis. Plant Physiol 1994;105: 35–45.

Tsuboi S, Osafune T, Tsugeki R, Nishimura M, Yamada M. Nonspecific lipid transfer protein in castor bean cotyledon cells: subcellular localization and a possible role in lipid metabolism. J Biochem (Tokyo) 1992;111: 500–8.

Wang SY, Wu JH, Ng TB, Ye XY, Rao PF. A non-specific lipid transfer protein with antifungal and antibacterial activities from the mung bean. Peptides 2004;25: 1235–42.

Wijaya R, Neumann GM, Condron R, Hughes AB, Polya GM. Defense proteins from seed of *Cassia fistula* include a lipid transfer protein homologue and a protease inhibitory plant defensin. Plant Sci 2000;159: 243–55.

Yang X, Li J, Li X, She R, Pei Y. Isolation and characterization of a novel thermostable non-specific lipid transfer protein-like antimicrobial protein from motherwort (*Leonurus japonicus Houtt*) seeds. Peptides 2006;27:3122–8.

Zachowski A, Guerbette F, Grosbois M, Jolliot-Croquin A, Kader J. Characterization of acyl binding by a plant lipid-transfer protein. Eur J Biochem 1998;257: 443–8.