An extracellular lipid transfer protein is relocalized intracellularly during seed germination

Luciana Pagnussat¹*, Christian Burbach², František Baluška² and Laura de la Canal¹,†

1 Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata-CONICET, Funes 3250, 7600 Mar del Plata, Argentina
2 IZMB, University of Bonn, Kirschellee 1, 53115 Bonn, Germany
* Current address: Facultad de Ciencias Agrarias-INTA Balcarce, Universidad Nacional de Mar del Plata, Ruta Nacional 226, Km. 73.5, 7620 Balcarce, Argentina
† To whom correspondence should be addressed. E-mail: ldelacan@mdp.edu.ar

Received 15 March 2012; Revised 5 October 2012; Accepted 10 October 2012

Abstract

Plant lipid transfer proteins (LTPs) constitute a family of small proteins recognized as being extracellular. In agreement with this notion, several lines of evidence have shown the apoplastic localization of HaAP10, a LTP from Helianthus annuus dry seeds. However, HaAP10 was recently detected intracellularly in imbibing seeds. To clarify its distribution, immunolocalization experiments were performed during the course of germination and confirmed its intracellular localization upon early seed imbibition. Further assays using a hydrophobic dye, FM4-64, inhibitors of vesicular traffic, and immunolocalization of the pectin rhamnogalacturonan-II, allowed the conclusion that endocytosis is activated as soon as seed imbibition starts. Furthermore, this study demonstrated that HaAP10 is endocytosed throughout imbibition. Biochemical and cellular approaches indicate that the intracellular fraction of this LTP appears associated with oil bodies and some evidence also suggest its presence in glyoxysomes. So, HaAP10 is apoplastic in dry seeds and upon imbibition is rapidly internalized and relocalized to organelles involved in lipid metabolism. The results suggest that HaAP10 may be acting as a fatty acid shuttle between the oil body and the glyoxysome during seed germination. This concept is consistent with the initial proposition that LTPs participate in the intracellular transfer of lipids which was further denied based on their apparent extracellular localization. This report reveals for the first time the relocalization of a lipid transfer protein and opens new perspectives on its role.

Key words: Extracellular, germination, Helianthus annuus, lipid mobilization, lipid transfer protein, oil storage.

Introduction

Lipid transfer proteins (LTPs) constitute a family of cationic peptides largely distributed in the plant kingdom (Carvalho and Gomes, 2007). Their denomination is due to the ability of LTPs to facilitate the transfer of phospholipids and fatty acids between artificial membranes in vitro (Kader, 1996). This observation was further validated by the detection of a hydrophobic binding cavity in the structure of several LTPs (Yeats and Rose, 2008). Immunochemical, tissue printing, and proteomic studies performed in diverse species have shown the extracellular localization of LTPs from barley (Skriver et al., 1992), Arabidopsis (Maldonado et al., 2002), tobacco (Dani et al., 2005), grape (Coutos-Thevenot et al., 1993), soybean (Djordjevic et al., 2007), and mungbean (Kusumawati et al., 2008), among others. Their targeting to the secretory pathway is also supported by the presence of a signal peptide in the amino terminus of all the LTPs studied so far. On the other hand, although LTPs are recognized as apoplastic proteins, some experimental evidence has shown that certain members of the family can be found intracellularly (Tsuboi et al., 1992; Carvalho et al., 2004; Diz et al., 2011).

The role of LTPs is still a matter of discussion, which is certainly a consequence of the large number of isoforms present in each plant, with different tissue and developmental expression patterns. As an example, at least 15 genes were originally detected in Arabidopsis (Arondel et al., 2000) and, according to
their annotation in databanks, the family could comprise much more members. LTPs have been implicated in several physiological roles such as wax assembly (Hollenbach et al., 1997), antimicrobial defence (Garcia-Olmedo et al., 1995), seed storage lipid mobilization (Tsiboi et al., 1992), pollen tube adhesion (Park and Lord, 2003), and plant signalling (Buhot et al., 2001). However, direct evidence on the function of LTPs has been only provided by a few reports so far. Hence, DEFECTIVE IN INDUCED RESISTANCE 1 is implicated in Arabidopsis defence signalling (Maldonado et al., 2002), a glycosylphosphatidylinositol-anchored LTP1 was shown to participate in cuticular wax deposition (DeBono et al., 2009), and Arabidopsis LTP5 plays a role in pollen tube tip growth and fertilization (Chae et al., 2009).

LTPs have been isolated from seeds of several species such as onion (Cammue et al., 1995), radish (Terras et al., 1992), maize (Sossountzov et al., 1991), wheat (Boutrot et al., 2005), chili pepper (Diz et al., 2011), cumin (Zaman and Abbasi, 2009), and coffee (Zottich et al., 2011), and have been characterized as antimicrobial peptides since they exert antifungal and/or antibacterial activity in vitro. A previous work (Regente and de la Canal, 2000) studied a LTP from sunflower (Helianthus annuus) seeds, HaAP10 (H. annuus antifungal protein 10kDa), which displays antifungal activity in vitro and is able to disturb phospholipid layers leading to fungal membrane permeabilization (Regente et al., 2005). This evidence is in accordance with the role of LTPs as extracellular antimicrobial peptides participating in seed defence against microorganisms. Surprisingly, Pagnussat et al. (2009) reported that HaAP10 shows an unexpected localization in germinating seeds using a biochemical approach: a fraction of the protein was found peripherically associated with microsomal and plasma membrane fractions. Moreover, immunochemical studies indicated that HaAP10 could be found intracellularly in imbibing seeds, associated with unidentified structures (Pagnussat et al., 2009).

The aim of this work was to clarify the localization of HaAP10 during the course of seed germination. Results presented here demonstrate that HaAP10 displays extracellular localization in dry seeds but, when imbibition starts, this LTP is rapidly targeted to intracellular oil mobilization-related structures. This finding constitutes a novel issue to understand the role of LTPs in seed physiology and germination, supporting the participation of these proteins in the mobilization of lipids during germination.

Materials and methods

Plant material

Helianthus annuus. L. seeds, line 10347, were kindly provided by Advanta Semillas (Argentina).

Steedman wax sections

Sunflower dry seeds or seeds imbibed in water for different times were decorticated, sectioned in 2 µm slices, and embedded in 10% dimethyl-sulphoxide (DMSO). Then the slices were subjected to a vacuum until boiling of the solution was reached (three times). DMSO was then replaced with fixative solution (0.05 M phosphate pH 7.4, 10% DMSO, 4% paraformaldehyde, 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 ethanol series as follows: 30% ethanol in phosphate buffer (PBS) 30 min; 50% ethanol 30 min; 70% ethanol 30 min; 90% ethanol 30 min; 96% ethanol 30 min. Samples were embedded in low-melting-point Steedman’s wax as previously described (Baluška et al., 1992), poured into plastic moulds and the wax was allowed to solidify. Sections (10 µm) were cut from these blocks using a rotary microtome, collected on 50% (w/v) polyethyleneimine-coated slides and dried for 24 h.

Fluorimmunolocalization and confocal microscopy

Seed sections were deparaffinated, rehydrated through ethanol series, and blocked as follows in 15-min steps: 100% ethanol, 96% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, PBS, and PBS with 3% bovine serum albumin (BSA). Labelling with HaAP10 (1:1000) (Pagnussat et al., 2009) and rhomnoaglacturonin II (RG-II; 1:200) (Matoh et al., 1998) antisemur, with secondary antibodies (1:500) Alexa 488-coupled goat anti-mouse IgG and Alexa 546-coupled goat anti-rabbit IgG, respectively, was carried out according to Gillespie et al. (2005). Microscopic analysis of immunofluorescence-labelled sections was performed using a Nikon C1 confocal laser scanning microscope. All images were acquired with a ×601/40/0.13 oil-immersion lens. Alexa 546-coupled antibody was excited at 543 nm and detected at 550–650 nm. N-(3,4-dihydroxyphenyl)alanine-diethylaminophenylhexatrienyl) pyridinium dibromide (FM4-64) was excited at 488 nm and detected at 650–750 nm. To avoid bleed-through in double-labelling experiments, all images were captured using line-sequential scanning mode. The post-processing of images was carried out with the aid of EZ-C1 FreeViewer version 3.2 software, Photoshop 6.0/CS, and Open Office applications.

Transmission electron microscopy

Sunflower imbibed seeds were cut and fixed as described above. After dehydration in graded ethanol series, the tissue was embedded in LR White Resin (hard grade; Biocell, Cardiff, UK) and left to polymerize at 36 °C. Ultra-thin sections were cut on an ultramicrotome and transferred onto Formvar-coated nickel grids. The sections were blocked with 50 mM glycine, 5% BSA, and 5% normal goat serum in PBS for 30 min and then washed with wash buffer (WB; 1% BSA and 0.1% gelatin in PBS). They were incubated initially with anti-HaAP10 (diluted 1:50 with WB) at room temperature for 90 min, washed with WB, and incubated with the secondary antibody, goat anti-mouse IgG-5 nm gold conjugate (diluted 1:50 in WB, Sigma-Aldrich) for 90 min. The sections were washed with WB and PBS, post-fixed with 3% glutaraldehyde for 15 min, washed extensively with distilled water, and contrasted with ice-cold 2% aqueous uranyl acetate and 1% osmium tetroxide. The labelled sections were examined in a LEO 912AB electron microscope (Zeiss, Oberkochen).

FM4-64 staining

Decorticated dry seeds were incubated with 50 µM FM4-64 (Molecular Probes) and 1 µM Sytox green (Molecular Probes) in PBS buffer and kept in the dark at 0 °C for 1 h. Afterwards, seeds were washed twice with PBS, sliced by hand, and immediately observed under laser scanning confocal microscope as mentioned above. As a positive control for cell death and Sytox green staining, a set of seeds were pre-treated with two vacuum pulses of 10% DMSO (Pagnussat et al., 2012).

Purification and protein analysis of oil bodies and glyoxysomes

Seeds imbibed for 24 h were decorticated and placed in cold 0.1 M potassium phosphate buffer (pH 7.2). All further procedures were carried out on ice. Seeds were homogenized with five parts (w/v) of 0.1 M potassium phosphate buffer containing 0.33 M sucrose, filtered through three layers of Miracloth, and centrifuged at 20,000 g for 20 min. Oil-bodies formed a white pad at the surface of the supernatant. After removal, the fat pad was mechanically dispersed in 50 mM TRIS-HCl buffer (pH 7.2) at a ratio of 60:1 (v/v) buffer to fat (Milichip et al., 1996). The Washing
Germination triggers internalization of a lipid transfer protein

The preparation was extracted with a 3-fold excess of diethyl ether to remove neutral lipids. Proteins were recovered by incubation with acetone 20 min at −20 °C and centrifugation for 15 min at 10,000 g.

Glyoxysomes were isolated using a standard protocol already performed in sunflower cotyledons (Jiang et al., 1994). Seeds imbibed for 1 h were homogenized in 1 M sucrose, 170 mM Tricine, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 0.9% (w/v) BSA, and 1 mM phenylmethanesulphonylfluoride (pH 7.5). The homogenate was filtered through three layers of cheesecloth and centrifuged for 10 min at 1500 g. The supernatant was centrifuged for 20 min at 14,000 g to obtain glyoxysome-enriched pellets. The pellets were gently suspended in 11 ml homogenizing medium. A 5.5 ml suspension of organelles was applied on top of a sucrose gradient composed of 4 ml of 60% (w/w), 5 ml of 57%, 7 ml of 51%, 5 ml of 47%, 5 ml of 42%, and 4 ml of 35% sucrose. Sucrose solutions were prepared in 1 mM EDTA, pH 7.5. The gradients were centrifuged for 1.5 h at 70,000 g. After centrifugation, gradient fractions were further evaluated for catalase activity as a marker protein (Jiang et al., 1994) and submitted to Western blotting to detect HaAP10.

SDS-PAGE was performed according to Laemmli (1970). The running gel was 12% acrylamide and the sample buffer contained 50 mM TRIS pH 6.8, 2% SDS, and 10% glycerol. For Western blotting, proteins were electroblotted into a 0.4-µm pore nitrocellulose membrane (20 min, 20 V; Trans-blot SD semidry transfer cell, Bio-Rad) in a buffer containing 39 mM glycine, 0.0376% SDS, 48 mM TRIS-base, and 20% methanol (final pH 9.2). Membranes were then incubated with blocking buffer (100 mM TRIS-HCl pH 8 and 1% BSA) for 30 min at room temperature and then with primary antibody diluted in blocking buffer. Antibodies were diluted as follows: 1:6000 for mouse anti-HaAP10 and 1:10,000 for rabbit anti-oleosins from Helianthus annuus (Beaudoin and Napier, 2000). Blots were washed and then incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies: anti-mouse IgG (Bio-Rad) at a concentration 1:5000 and anti-rabbit IgG (Bio-Rad) at a concentration 1:10,000 in blocking buffer. Signal detection was performed as described elsewhere (Regente and de la Canal, 2000).

Results

Changes in HaAP10 localization during seed imbibition

The extracellular localization of HaAP10 in dry seeds was supported by biochemical approaches (Regente and de la Canal, 2000) and the presence of a signal peptide in its cDNA (Regente and de la Canal, 2003), but microscopic validation was lacking. Therefore, immunolocalization under confocal microscope was performed using a specific HaAP10 antibody. Since previous evidence has shown HaAP10 accumulation in cotyledons (Gonorazky et al., 2005), the analysis was focused to this region of the seeds. Immunolocalization experiments confirmed the presence of the LTP in the extracellular space of cotyledon parenchymatic cells (Fig. 1A), while bright-field showed a profuse distribution of oil bodies inside the cells (Fig. 1B). In order to evaluate possible changes in HaAP10 localization during seed rehydration, fluorimmunolocalization experiments were performed at different stages of imbibition. It can be seen that, while HaAP10 exhibited an exclusively apoplastic localization in dry seeds (Fig. 2A), after only 20 min of imbibition the protein started to be detected inside the cells (Fig. 2B). This localization

---

**Fig. 1.** Immunolocalization of HaAP10 in cotyledons of sunflower dry seeds. (A) Confocal laser scanning microscopy showing immunofluorescence labelling of HaAP10. (B) Merge images of fluorescence and bright-field of section A. Bar, 20 µm (this figure is available in colour at JXB online).

**Fig. 2.** Immunolocalization of HaAP10 during seed imbibition. Confocal laser scanning microscopy showing HaAP10 localization in dry seed cotyledon cells (A) or after 20 min (B), 2 h (C), and 16 h (D) of water imbibition. Bars, 20 µm (this figure is available in colour at JXB online).
was more evident after 2 and 16 h of imbibition (Fig. 2C, D). This fast change in HaAP10 localization triggered by seed imbibition, in addition to the recent description of endocytosis during early germination in Arabidopsis (Pagnussat et al., 2012), prompted this study to investigate whether endocytic events could be responsible for the appearance of this LTP inside the cells.

HaAP10 is endocytosed during imbibition

FM4-64 dyes are widely used to study endocytosis and vesicle trafficking in living eukaryotic cells. Because of their amphipathic nature, FM dyes are unable to cross membranes and anchor to the outlet leaflet of the bilayer (Bolte et al., 2004). To determine if endocytosis takes place during imbibition of sunflower seeds, dry seeds were incubated in the presence of FM4-64 and dye internalization was clearly observed 1 h after the beginning of imbibition (Supplementary Fig. S1, available at JXB online). Absence of Sytox Green fluorescence in the nucleus of the cells indicated that they were alive. Thus, the intracellular FM4-64 signal was not a consequence of cell death but rather an endocytic process operating during sunflower seed germination.

The vesicle trafficking inhibitor brefeldin A (BFA) is extensively used to analyse endocytosis. In plants, treatment with this inhibitor leads to the formation of large ‘BFA-induced compartments’ in cells that accumulate internalized macromolecules via retrograde endocytic pathways (Peyroche et al., 1999; Lam et al., 2009). This response constitutes a valuable tool to assess if a molecule is actively internalized by endocytosis (Baluška et al., 2002) and so was used to analyse HaAP10 internalization through fluorimmunolocalization. Fig. 3 shows that HaAP10 had a diffuse intracellular localization after 1 h of water imbibition (Fig. 3A) while upon BFA treatment the protein changed its localization, being detected in apparent BFA-induced compartments (Fig. 3B). In fact, the same behaviour was observed for rhamnogalacturonan II (RG-II; Fig. 3C, D), a complex pectic polysaccharide present in plant cell walls whose endocytosis has been demonstrated in other species (Yu et al., 2002; Baluška et al., 2005). RG-II constitutes an excellent tool to analyse endocytic events since an antibody has been raised that only recognizes the extracellularly assembled borate–RGII complex internalized from the extracellular compartment (Matoh et al., 1998). Fluorimmunolocalization experiments showed that in dry sunflower seeds, RG-II epitopes were mainly localized in cell walls (Supplementary Fig. S2). On the other hand, in imbibed seeds this pectin was also observed inside the cells (Fig. 3C), indicating that RG-II may be endocytosed upon imbibition. Furthermore, when sunflower seeds were treated for 1 h with 5 µM BFA, RG-II accumulated in intracellular BFA-induced compartments (Fig. 3D). Merge images between immunodetected RG-II and HaAP10 confirmed a strong intracellular co-localization of both epitopes in BFA-induced compartments (yellow in Fig. 3F). All together, these results indicate that HaAP10 is actively internalized by endocytosis upon early imbibition of sunflower seeds.

As a first approach to understand the mechanism operating in HaAP10 endocytosis, this study evaluated the effect of wortmannin, an inhibitor of phosphatidylinositol-3-kinase which has been shown to interfere with endocytic processes (Reichardt et al., 2007; Ebine et al., 2011). Moreover, a recent report showed that wortmannin treatment causes aggregation of clathrin at the plasma membrane, which might explain its inhibitory effect on endocytosis (Ito et al., 2012). Imbibition of sunflower seeds for 1 h in the presence of 33 µM wortmannin resulted in HaAP10 retention in the plasma membrane instead of appearing intracellularly (Supplementary Fig. S3). Although additional evidence is required to unequivocally identify the endocytosis mechanism, the retention of HaAP10 in the plasma membrane upon wortmannin treatment suggests that it could be endocytosed via a clathrin-dependent pathway.

Intracellular localization of HaAP10

The intracellular distribution of HaAP10 observed in Fig. 2, taken in conjunction with the ability of LTPs to interact with fatty acid chains, is a reminder of the originally proposed role for LTPs: intracellular lipid transfer. Since sunflower seeds contain a large number of oil bodies where triacylglycerides (TAGs) are stored, this study explored whether HaAP10 is associated with these organelles in seeds submitted to imbibition. Oil bodies are intracellular compartments composed of a TAG matrix surrounded by a phospholipid monolayer stabilized by basic proteins called oleosins (Tzen et al., 1992). As a first approach to precisely determine the intracellular localization of HaAP10, oil bodies were isolated as previously described for sunflower seeds (Millichip et al., 1996) and fractionated by SDS-PAGE. Western blotting analysis using antibodies raised against sunflower oleosins clearly detected the marker protein in the oil body-enriched fraction (Fig. 4). Interestingly, anti-HaAP10 serum revealed the presence of the LTP in this fraction (Fig. 4), suggesting that it could be associated with oil bodies. In order to confirm the association of HaAP10 with oil bodies, a cellular approach based on co-immunolocalization between HaAP10 and oleosins was performed. As expected, in dry seeds both epitopes remained at different localizations: HaAP10 extracellularly (green, Fig. 5A) and oleosins in the oil bodies (red, Fig. 5A). However, upon imbibition HaAP10 and oleosins showed a strong co-localization (yellow, Fig. 5B), which indicates that HaAP10 may be found in oil bodies from imbibed seeds. Labelling with preimmune serum (Fig. 5C) or secondary antibody alone (Fig. 5D) did not show any fluorescence, confirming that the signal observed with HaAP10 antibodies is specific. In conclusion, both cellular and biochemical approaches showed that HaAP10 is localized in oil bodies during germination. However, due to the extensive compacting of sunflower seed cells, care must be taken in determining the identity of the intracellular compartments with which HaAP10 is associated. Therefore, TEM was also used to enhance the resolution and confirm the subcellular localization of HaAP10. Immunolocalization experiments using TEM with secondary gold-conjugated antibodies confirmed that HaAP10 was localized in oil bodies (Fig. 6) and also seemed to be present associated with glyoxysomes (Fig. 6A). Since TEM observations were not conclusive proof of the presence of HaAP10 in glyoxysomes, glyoxysome enriched fractions were isolated and examined for HaAP10 presence by Western blotting. Glyoxysome enrichment through a sucrose discontinuous
Germination triggers internalization of a lipid transfer protein

gradient (Jiang et al., 1994) revealed that HaAP10 co-localized with catalase (glyoxysome marker) at the bottom of the gradient (Supplementary Fig. S4, fractions 1–4), but was also detected throughout the entire gradient, consistent with its...
localization in other compartments. Even though these results are not conclusive, they support the presence of HaAP10 in glyoxysomes.

Discussion

This paper provides evidence demonstrating that HaAP10, a seed-specific lipid transfer protein, is localized in the apoplast of dry seeds but changes its distribution when imbibition starts, being also associated with oil bodies. In fact, rather fast relocalization of HaAP10 was observed, that became detectable only 20 minutes after the start of seed rehydration. The experiments performed indicate that active endocytosis operates during imbibition and is responsible for HaAP10 internalization. The endocytosis of HaAP10 may explain previous results showing that a fraction of HaAP10 appeared associated with the plasma membrane of germinating seeds (Pagnussat et al., 2009). Although the mechanisms determining HaAP10 endocytosis are still unknown, this novel concept of relocalization upon imbibition sounds physiologically plausible. In fact, developing seeds are known to accumulate a large number of proteins and RNA, which constitute a rapid supply of metabolites when activation of quiescent dry seeds takes place. Likewise, in different physiological situations, re-localization of plant proteins results in a fast availability of proteins that have been strategically stored in other compartments, as already described for RNA-binding proteins (Campalans et al., 2004), a cysteine protease (Bernoux et al., 2008), or ALY proteins (Uhrig et al., 2004), among others.

The current observations constitute the first demonstration of the re-localization of a LTP and the meaning of this change must be analysed in detail. First, the exclusive apoplastic localization of HaAP10 in dry seeds is consistent with observations previously reported. These include the fact that the protein is extracted from apoplastic fluids and is even recovered in the imbibition milieu (Regente and de la Canal, 2000). In this sense, and taking into account the potent antimicrobial activity and its ability to permeabilize fungal cells (Regente et al., 2005), the previously suggested role as a defence protein cannot be excluded. Concerning HaAP10 subcellular localization in imbibing seeds, three lines of evidence indicate that it is associated with oil bodies. First, this study shows that HaAP10 co-purifies with oil bodies. Second, HaAP10 co-localizes with oleosins under confocal microscopy. Third, immunolocalization of HaAP10 and observation under TEM demonstrated its association with oil bodies. On the other hand, the apparent
association of HaAP10 with glyoxysomes is suggested by TEM observations and co-purification with catalase enriched fractions, but remains to be confirmed. The dual localization of HaAP10 (extra- and intracellular) suggests that the protein may account for different functions in the seeds according to their physiological state.

LTPs were discovered when demonstrating the ability of a protein isolated from potato tubers to transfer phospholipids between membranes in vitro (Kader, 1975). Thereon, other LTPs were isolated displaying the same properties and, thus, LTPs have been originally proposed to play a major role in the intracellular movement of lipids. However, a major constraint for that role was the detection of several LTPs located extracellularly or their secretion inferred by the presence of a signal peptide in the deduced protein sequence (reviewed in Kader, 1997). In this context, a possible role of LTPs in the intracellular lipids dynamics became unlikely and in the past years the functions assigned to members of the LTP family were related to their extracellular location. Interestingly, this paper shows, as far as is known for the first time, that a LTP is endocytosed, and this change in localization may have relevant consequences related to its function. According to the current knowledge, it can be speculated that during imbibition HaAP10 is relocated to oil bodies (and probably to glyoxysomes) to participate in the degradation of TAGs triggered during seed germination. TAGs are the major seed storage reserve in many plant species including oil crops such as sunflower and soybean. They accumulate into oil bodies during seed maturation and are stored until mobilization of reserves is required to sustain seedling growth (Huang, 1992; Nonogaki et al., 2000). During lipid mobilization, oil body lipases hydrolyse TAGs to produce free fatty acids and glycerol. Those fatty acids enter the glyoxysome where β-oxidation and part of the glyoxylate cycle occurs (Penfield et al., 2005; Graham, 2008). Despite this being a well-known pathway, the translocation mechanism used by fatty acids to reach glyoxysomes still remains elusive, even though some evidence indicate that ABC transporters may contribute to the translocation of certain fatty acids (Hayashi et al., 2001; Footitt et al., 2002).

Although the extracellular localization of LTPs negated their initially proposed role in intracellular lipid transfer, the current results demonstrate that HaAP10 relocalizes to intracellular organelles involved in lipid metabolism and may play the originally suggested function. Since some seed LTPs have been also detected intracellularly (Tsuboi et al., 1992; Carvalho et al., 2004; Diz et al., 2011), this report opens new perspectives on the physiological role of these LTPs. This study postulates the internalization of the LTP HaAP10 to participate in the transfer of fatty acids to the glyoxysome. Work is in progress to elaborate on this hypothesis further.

**Supplementary material**

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Internalization of FM4-64 during early imbibition of sunflower.

Supplementary Fig. S2. Rhamnogalacturonan II is extracellularly localized in sunflower dry seeds.

Supplementary Fig. S3. HaAP10 retention in the plasma membrane upon Wortmannin treatment.

Supplementary Fig. S4. HaAP10 is present in glyoxysome fractions.

**Acknowledgements**

This work was funded by the ANPCYT, CONICET, and the University of Mar del Plata, Argentina. The authors thank Dr. Beaudoin and Dr. Toru Matoh for providing the oleosin and RG-II antisera, respectively. L.P. acknowledges Martina Beck for assistance with confocal scanning microscopy and Ursula Mettbach for assistance with TEM. L.D.L.C and L.P. are a researcher and a doctoral fellow, respectively, from CONICET. L.P. was a recipient of a European Molecular Biology Organization short-term fellowship at the IZMB.

**References**


Germination triggers internalization of a lipid transfer protein


