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RESEARCH PAPER

An extracellular lipid transfer protein is relocalized intracellularly during seed germination

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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 *Ha*AP10, a LTP from *Helianthus annuus* dry seeds. However, *Ha*AP10 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 *r*hamnogalacturonan-II, allowed the conclusion that endocytosis is activated as soon as seed imbibition starts. Furthermore, this study demonstrated that *Ha*AP10 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, *Ha*AP10 is apoplastic in dry seeds and upon imbibition is rapidly internalized and relocalized to organelles involved in lipid metabolism. The results suggest that *Ha*AP10 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

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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 (García-Olmedo *et al.*, 1995), seed storage lipid mobilization (Tsuboi *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), chilli 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, *Ha*AP10 (*H. annuus* antifungal protein 10 kDa), 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 *Ha*AP10 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, immunolocalization studies indicated that *Ha*AP10 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 *Ha*AP10 during the course of seed germination. Results presented here demonstrate that *Ha*AP10 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% dimethylsulphoxide (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) polyethylenimine-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 *Ha*AP10 (1:1000) (Pagnussat *et al.*, 2009) and rhamnogalacturonan II (RG-II; 1:200) (Matoh *et al.*, 1998) antiserum, 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 ×60/1.40/0.13 oil-immersion lens. Alexa 546-coupled antibody was excited at 543 nm and detected at 550–650 nm. N-(3-triethylammoniumpropyl)-4-(6-(4-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-*Ha*AP10 (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 (Millichip *et al.*, 1996). The Washing

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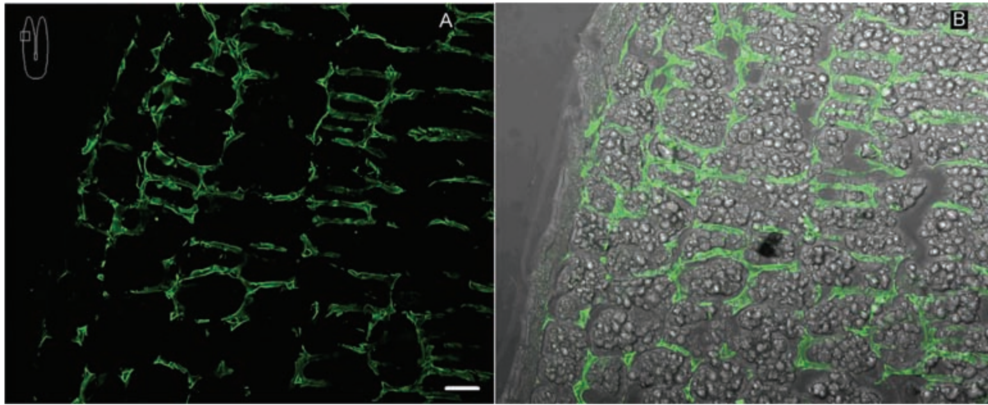

Fig. 1. Immunolocalization of *Ha*AP10 in cotyledons of sunflower dry seeds. (A) Confocal laser scanning microscopy showing immunofluorescence labelling of *Ha*AP10. (B) Merge images of fluorescence and bright-field of section A. Bar, 20 µm (this figure is available in colour at *JXB* online).

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procedure was repeated three times. 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 $MgCl_2$, 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 *Ha*AP10.

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-*Ha*AP10 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 Ha*AP10 localization during seed imbibition*

The extracellular localization of *Ha*AP10 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 *Ha*AP10 antibody. Since previous evidence has shown *Ha*AP10 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 *Ha*AP10 localization during seed rehydration, fluorimmunolocalization experiments were performed at different stages of imbibition. It can be seen that, while *Ha*AP10 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

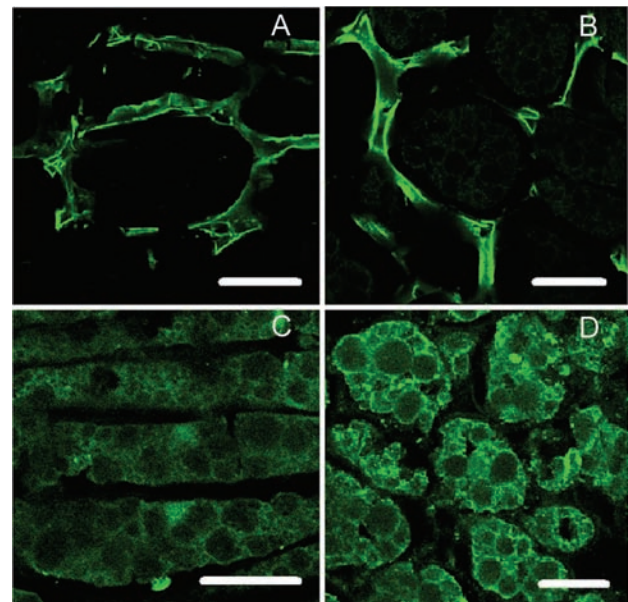

Fig. 2. Immunolocalization of *Ha*AP10 during seed imbibition. Confocal laser scanning microscopy showing *Ha*AP10 localization in dry seed cotyledon cells (A) or after 20 min (B), 2 h (C), and 16 h (D) of water imbibition. Bars, 20 µm (this figure is available in colour at *JXB* online).

was more evident after 2 and 16 h of imbibition (Fig. 2C, D). This fast change in *Ha*AP10 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.

*Ha*AP10 *is endocytosed during imbibition*

FM4-64 dyes are widely used to study endocytosis and vesicle trafficking in living eukaryotic cells. Because of their amphiphilic 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 *Ha*AP10 internalization through fluorimmunolocalization. Fig. 3 shows that *Ha*AP10 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 *Ha*AP10 confirmed a strong intracellular co-localization of both epitopes in BFA-induced compartments (yellow in Fig. 3F). All together, these results indicate that *Ha*AP10 is actively internalized by endocytosis upon early imbibition of sunflower seeds.

As a first approach to understand the mechanism operating in *Ha*AP10 endocytosis, this study evaluated the effect of wortmannin, an inhibitor of phosphatidylinositiol-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 *Ha*AP10 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 *Ha*AP10 in the plasma membrane upon wortmannin treatment suggests that it could be endocytosed via a clathrin-dependent pathway.

Intracellular localization of *Ha*AP10

The intracellular distribution of *Ha*AP10 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 *Ha*AP10 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 *Ha*AP10, 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-*Ha*AP10 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 *Ha*AP10 with oil bodies, a cellular approach based on co-immunolocalization between *Ha*AP10 and oleosins was performed. As expected, in dry seeds both epitopes remained at different localizations: *Ha*AP10 extracellularly (green, Fig. 5A) and oleosins in the oil bodies (red, Fig. 5A). However, upon imbibition *Ha*AP10 and oleosins showed a strong co-localization (yellow, Fig. 5B), which indicates that *Ha*AP10 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 *Ha*AP10 antibodies is specific. In conclusion, both cellular and biochemical approaches showed that *Ha*AP10 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 *Ha*AP10 is associated. Therefore, TEM was also used to enhance the resolution and confirm the subcellular localization of *Ha*AP10. Immunolocalization experiments using TEM with secondary gold-conjugated antibodies confirmed that *Ha*AP10 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 *Ha*AP10 in glyoxysomes, glyoxysome enriched fractions were isolated and examined for *Ha*AP10 presence by Western blotting. Glyoxysome enrichment through a sucrose discontinuous

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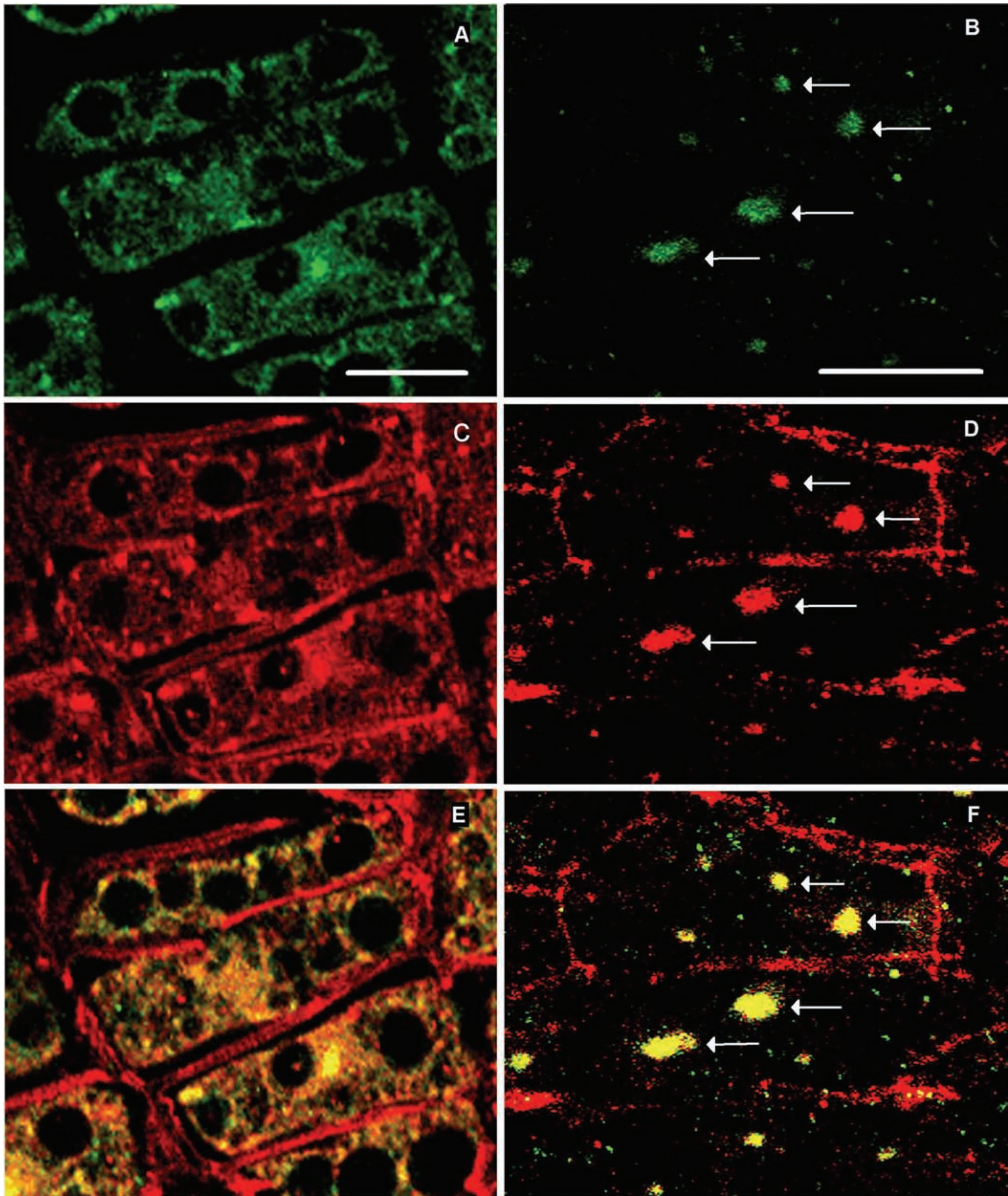

Fig. 3. *Ha*AP10 is endocytosed during imbibition and accumulates in BFA-induced compartments. Confocal laser scanning microscopy showing immunolocalization of *Ha*AP10 (green; A, B), rhamnogalacturonan II (red; C, D), and merge image (E, F). Seeds were imbibed for 1 h in water (A, C, and E) or in 5 μM brefeldin A (B, D, and F) and cotyledons were prepared for immunolocalization. Arrows indicate BFA-induced compartments. Bars, 20 μm.

gradient (Jiang *et al.*, 1994) revealed that *Ha*AP10 co-localized with catalase (glyoxysome marker) at the bottom of the gradient (Supplementary Fig. S4, fractions 1–4), but was also detected thorough the entire gradient, consistent with its

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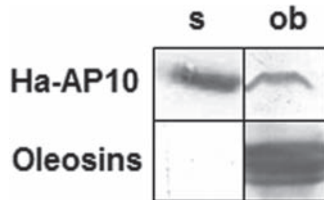

Fig. 4. *Ha*AP10 is present in oil body fractions. Immunodetection of *Ha*AP10 and oleosins in total soluble (s) and oil body (ob) protein fractions. Proteins were extracted as detailed in Materials and methods and analysed by SDS-PAGE followed by immunoblotting.

localization in other compartments. Even though these results are not conclusive, they support the presence of *Ha*AP10 in glyoxysomes.

Discussion

This paper provides evidence demonstrating that *Ha*AP10, 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 *Ha*AP10 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 *Ha*AP10 internalization. The endocytosis of *Ha*AP10 may explain previous results showing that a fraction of *Ha*AP10 appeared associated with the plasma membrane of germinating seeds (Pagnussat *et al.*, 2009). Although the mechanisms determining *Ha*AP10 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 *Ha*AP10 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 *Ha*AP10 subcellular localization in imbibing seeds, three lines of evidence indicate that it is associated with oil bodies. First, this study shows that *Ha*AP10 co-purifies with oil bodies. Second, *Ha*AP10 co-localizes with

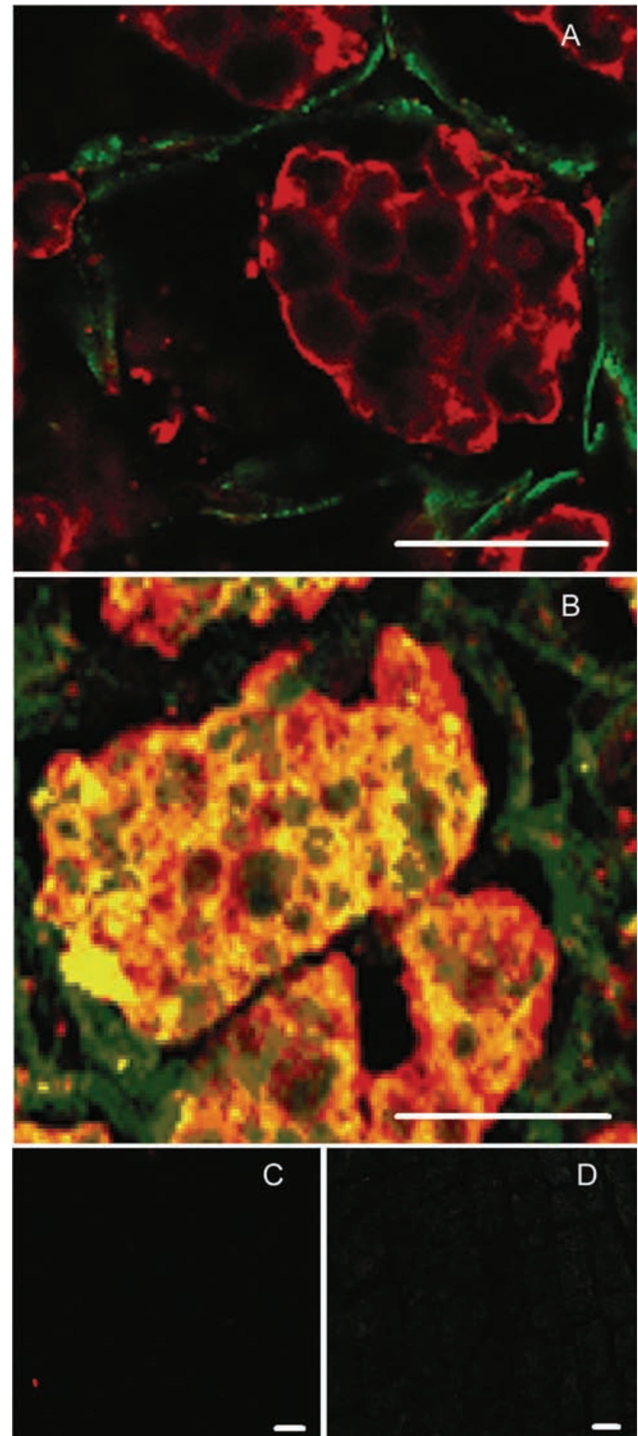

Fig. 5. *Ha*AP10 shows a strong co-localization with oleosins in imbibed seeds. Confocal laser scanning microscopy merge images of co-immunolocalization experiments between *Ha*AP10 (green) and oleosins (red) in dry (A) or 1 h imbibed (B) cotyledons of sunflower seeds. C and D show control assays using preimmune serum or both secondary antibodies respectively. Bars, 20 µm.

oleosins under confocal microscopy. Third, immunolocalization of *Ha*AP10 and observation under TEM demonstrated its association with oil bodies. On the other hand, the apparent

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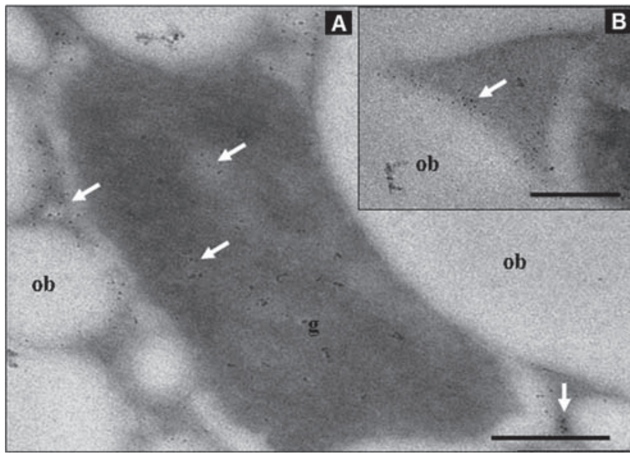

Fig. 6. TEM confirms the presence of *Ha*AP10 in oil bodies. (A) Micrograph showing immunogold labelling of *Ha*AP10 in cotyledon cells of 1 h water imbibed seeds in apparent glyoxysomes (g) and oil bodies (ob). (B) Micrograph of *Ha*AP10 localization in oil bodies. Arrows indicate the gathering of gold particles. Bar, 200 nm.

association of *Ha*AP10 with glyoxysomes is suggested by TEM observations and co-purification with catalase enriched fractions, but remains to be confirmed. The dual localization of *Ha*AP10 (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 *Ha*AP10 is relocalized 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 *Ha*AP10 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 *Ha*AP10 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. *Ha*AP10 retention in the plasma membrane upon Wortmannin treatment.

Supplementary Fig. S4. *Ha*AP10 is present in glyoxysome fractions.

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