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RESEARCH PAPER

New insights into the early steps of oil body mobilization during pollen germination

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Abstract

In some plants, pollen grains accumulate storage lipids that serve as energy supply during germination. Here, three enzymes involved in early steps of oil body mobilization in the male gametophyte were functionally characterized for the first time. The effect of extracellular sugars on pollen performance and oil body dynamics was also analysed. Olive pollen oil bodies showed phospholipase A, lipase, and lipoxygenase activities on their surface. Enzyme activity levels increased during germination with a maximum after 3 h. Removal of extracellular sugars from the germination medium did not affect pollen performance but increased enzyme activity rates and sped up oil body mobilization. Inhibitors seriously hampered pollen germination and pollen tube growth, leading to a characteristic accumulation of oil bodies in the germinative aperture. It can be concluded that storage lipids are sufficient for proper olive pollen germination. A lipase and a lipoxygenase are likely involved in oil body mobilization. Extracellular sugars may modulate their function, while a phospholipase A may promote their access to the storage lipids.

Key words: Lipase, lipoxygenase, oil body, olive, phospholipase A, pollen germination.

Introduction

In oleaginous plants, storage neutral lipids serve as energy supply and provide carbon equivalents for periods of active metabolism (Murphy, 2012). Storage lipids can be found in different sporophytic plant organs and tissues such as oilseeds and oleaginous fruits (Huang, 1994; Zienkiewicz *et al*., 2011a), as well as in cells of both male (Piffanelli *et al*., 1998) and female (Jiang *et al*., 2009) germ lines. In plant seeds, neutral lipids are stored in specialized organelles called oil bodies. Despite they have often been regarded as simple storage sites, a plethora of unsuspected functions has been suggested for these organelles, including subcellular lipid trafficking and turnover, and calcium signalling (Murphy, 2012).

Oil bodies consist of a triacylglycerol (TAG) matrix surrounded by a monolayer of phospholipids, in which a set of a few unique proteins are embedded (Huang, 1994). The disruption of this barrier may be required to promote access of degrading enzymes to the storage TAGs. In a pioneer study, Matsui *et al*. (1999) found that trypsin-assisted *in vitro* digestion of oil body-associated proteins led to oxygenation of TAGs by the action of a specific lipoxygenase (LOX) enzyme in cucumber cotyledons. More recently, it was reported that a patatin-type phospholipase promotes the LOX-dependent oxygenation of oil body phospholipids in cucumber cotyledons (Rudolph *et al*., 2011). Thus, phospholipase A (PLA) effectively produces 80-nm holes in the phospholipid monolayer (Noll *et al*., 2000).

The biochemical machinery and the metabolic pathways involved in conversion of storage TAGs to sugars during seed germination has been extensively studied over the last 50 years (Graham, 2008; Murphy, 2012). It is widely accepted that fatty acids are initially released from TAGs and transported to glyoxysomes, where they are activated to their acyl-CoA esters and degraded via β-oxidation. The *SUGAR-DEPENDENT1* (*SDP1*) gene encodes a bona-fide TAG lipase that contains a patatin-like domain and is involved in TAG breakdown (Eastmond, 2006). An alternative pathway of TAG mobilization, which is dependent of a specific LOX enzyme, may occur in parallel in some oilseeds (Feussner *et al*., 1994). This LOX enzyme catalyses oxygenation of linoleate moieties (18:2) of oil body-derived TAGs to (9Z,11E,13S)-13-hydroperoxy octadeca-9,11-dienoic acid (13-HPOD) (Gerhardt *et al*., 2005). 13-HPOD is then released from TAGs, reduced to (9Z,11E,13S)-13-hydroxy octadeca-9,11-dienoic acid (13-HOD) by the peroxygenase activity of caleosin (Hanano *et al*., 2006), and degraded via the β-oxidation spiral.

The pollen grain is the second most active site, after the seed, in TAG biosynthesis (Piffanelli *et al*., 1998). The mature olive pollen grain accumulates a high number of oil bodies in the cytoplasm of the vegetative cell (Rodríguez-García *et al*., 2003; Zienkiewicz *et al*., 2011b). During pollen germination, these organelles polarize near the germinative aperture and move to the emerging pollen tube (Rodríguez-García *et al*., 2003). Storage lipids are progressively metabolized during further steps of pollen tube growth (Zienkiewicz *et al*., 2010). These data suggest that oil bodies might provide the pollen tube with energy for a rapid apical growth. However, despite the importance of storage lipids for the growing of the pollen tube, little is known about the enzyme machinery involved in storage lipid mobilization in the male gamethophyte. Here, this study functionally characterizes for the first time three enzymes, namely lipase, PLA, and LOX, involved in early steps of oil body mobilization in the olive (*Olea europaea* L.) pollen. It also analyses the effect of extracellular sugars on pollen performance and oil body dynamics during germination and pollen tube growth.

Materials and methods

Plant material

Olive (*Olea europaea* L. cv. Picual) pollen grains were harvested as previously described (Zienkiewicz *et al*., 2010). Pollen samples were germinated according to Zienkiewicz *et al.* (2010) in a liquid culture medium with [(+)Su] or without [(–)Su] 10% (w/v) sucrose. Germinated pollen grains were sampled after 1, 3, 6, and 12 h of culture, and the germination rate (%) was calculated as previously described (Rejón *et al.*, 2012).

Oil body morphometry

Oil bodies were isolated from olive mature and germinated pollen [1, 3, and 6 h in either (+)Su or (–)Su medium], stained with Nile red, and their number and size (i.e. the largest diameter) were recorded as described by Zienkiewicz *et al.* (2010).

Protein extraction

Oil body-associated proteins were extracted as described by Zienkiewicz *et al.* (2010). Mature and germinated pollen [1, 3, and 6 h in (+)Su medium] samples were ground in N_2 to a very fine powder using a mortar and pestle and resuspended in 1.5 ml of 0.05 M phosphate buffer (pH 7.0). Total proteins were eluted under continuous stirring at 4 °C for 1 h. Protein suspensions were clarified by centrifugation at 13,500 *g* for 30 min at 4 °C and the resulting supernatants were stored at –20 °C until use. The protein concentration was measured using the 2D Quant Kit (Amersham Biosciences, USA) following the manufacturer's instructions.

In vitro *assays of lipase and PLA activity*

Proteins were extracted from germinated pollen (1, 3, and 6 h) grown in (+)Su and (–)Su medium, as described above. For lipase activity, 50 µl protein extract (~50 µg of protein) were incubated with 10 µl of 25 µg ml^{-1} resorufin ester (Sigma-Aldrich, USA) for 10 min, and the absorbance was read at A_{550} in an iMark Microplate Reader (Bio-Rad, USA). The PLA activity was assayed at A_{488} using 10 µl of 1 mM BODIPY FL C_{11}-PC (Molecular Probes, USA) as substrate. Control reactions were performed by omitting protein extracts in the reaction mixture.

Western blot analysis

Proteins from mature and germinated pollen [3 h in (+)Su medium] were electroblotted as previously described (Zienkiewicz *et al*., 2010). Immunodetection of LOX was performed by incubation with a polyclonal anti-*Glycine max* (soybean) LOX antibody (Agrisera, Sweden), diluted 1:1000 in TBS buffer (pH 7.2) containing 1% (w/v) bovine serum albumin (BSA) overnight at 4 °C, followed by a DyLight 488-conjugated anti-rabbit IgG secondary antibody (Agrisera), diluted 1:2000 in TBS for 2 h. The signal was detected in a Pharos FX imager (Bio-Rad).

In-gel assays of lipase and LOX activities

Lipase activity was assayed in gel using β-naphthyl palmitate as substrate, as previously described (Rejón *et al.*, 2012). LOX activity was measured according to Heinish *et al.* (1996), with minor modifications. Briefly, the gel was incubated for 30 min in a solution containing either α-linolenic acid or a mixture of α-linolenic acid and 10 mM sodium cyanide. Subsequently, the gel was stained with 100 ml of a solution containing 0.5 g of *N*,*N*-dimethyl-*p*-phenylenediamine, 4.5 ml methanol, and 0.5 ml acetic acid. Control reactions were performed by excluding the substrate.

Lipase and LOX inhibition assays

Pollen samples were germinated in either (+)Su or (–)Su medium supplemented with the corresponding inhibitor at a final concentration of 2 mM. Pollen was sampled after 3 h of culture and the germination rate (%) was calculated as described above. Ebelactone B (dissolved in ethanol/H_2O, 1:1) and ferulic acid (prepared in ethanol) were purchased from Sigma-Aldrich. Controls were carried without inhibitors.

To perform in-gel inhibition assays, protein extracts from germinated pollen [3 h in (+)Su medium] were prepared as above but the extraction buffer contained 2 mM ferulic acid. After SDS-PAGE, LOX activity was detected as described above. Reproducibility of results was confirmed in three independent experiments.

LOX immunoprecipitation and mass spectrometry analysis

Proteins were extracted from oil bodies as described above. LOX immunoprecipitation was carried out using the anti-soybean LOX antibody, as described by Zienkiewicz *et al.* (2010). After electrophoresis, the CBB-stained LOX band was sliced and subjected to MS/MS analysis. The identification of olive pollen LOX was carried out at the Laboratory of Proteomics (CSIC/UAB), a member of ProteoRed network (www.proteored.org). Samples were analysed using a linear LTQ ion trap equipped with a microESI ion source (ThermoFisher, USA). MS/MS spectra were analysed using the PEAKS Studio v5.1 software (Bioinformatics Solutions, Canada). Sequence tags with a confidence higher than 70%

were sent for protein identification against Uniprot database (taxonomy: 3193 embryophyta, release 15.15) using the Basic Local Alignment Search Tool (BLAST). Only the sequence tags with more than 80% of coincidence with the identified protein were considered. The identifications were manually validated to ensure the quality of the spectral data.

In situ *localization of lipase and PLA activity*

Oil bodies were isolated from germinated pollen [3 h in (+)Su medium] and incubated with either an aqueous solution of 25 µg ml^{-1} resorufin ester (lipase substrate) or an ethanolic solution of 1 mM BODIPY FL C_{11}–PC 9 (PLA substrate). *In situ* localization of lipase and PLA activity was also carried out in intact pollen tubes. Samples were incubated in either 25 µg resorufin ester ml^{-1} for 30 min or 1 mM BODIPY FL C_{11}-PC for 10 min, and examined with a C1 confocal laser scanning microscope (Nikon, Japan) using a He-Ne (549 nm) and an argon (488 nm) laser, respectively.

Light microscopy immunolocalization of LOX

LOX was immunolocalized on intact pollen tubes and isolated oil bodies [3 h in (+)Su medium]. Oil body samples were processed as described by Zienkiewicz *et al.* (2010) and incubated with the anti-soybean LOX antibody (diluted 1:30 in PBS buffer, pH 7.2, containing 1% BSA) for 3 h, followed by incubation with an anti-rabbit IgG DyLight 488-conjugated secondary antibody (diluted 1:200 in PBS), for 1 h. Germinated pollen samples were processed as above but the primary antibody was diluted 1:50 and incubation was carried out overnight at 4 °C. All the samples were re-suspended in an anti-fading solution of Citifluor (Sigma-Aldrich) and examined in a C1 confocal microscope using an argon (488 nm) laser. Ten µl of 0.1 mg Nile red ml^{-1} were added to the germinated pollen samples before observation. Z-series images were collected using the EZ-C1 Gold version 2.10 software (Nikon). Negative controls were prepared by excluding the primary antibody.

Transmission electron microscopy immunolocalization of LOX

Germinated pollen [3 h in (+)Su medium] samples were processed for transmission electron microscopy as previously described (Zienkiewicz *et al.*, 2010). Sections were incubated with an anti-soybean LOX antibody (diluted 1:50 in PBS buffer, pH 7.2, containing 0.5% BSA), overnight at 4 °C, followed by an anti-rabbit IgG 15-nm gold-conjugated secondary antibody (British Biocell International, UK), diluted 1:100 in PBS, for 1 h. After washing, sections were stained with 5% (w/v) uranyl acetate for 30 min. Samples were observed in a JEM-1011 transmission electron microscope (JEOL, Japan) operating at 80 kV. Control reactions were carried out by omitting the primary antibody.

Statistical analysis

Standard analysis of variance (t-test) was used to assess the significance of the means at $P < 0.01$ level using the SSPS Statistics version 19 software (IBM, USA).

Results

Olive pollen oil bodies' behaviour in sucrose-supplemented vs. sucrose-free culture medium

This study first compared germination and pollen tube growth rates in (+)Su vs. (–)Su cultured pollen. Germination rates were slightly lower in (–)Su medium (Fig. 1A and Supplementary Fig. S1, available at *JXB* online). Furthermore, there were slight differences in the average pollen tube length between (+)Su and (–)Su germination media at the early stages of culture (Fig. 1B).

Nile red staining allowed visualization of oil bodies inside pollen as fluorescent round-shaped structures (Supplementary Fig. S2). After 1 h of culture, a single pollen grain contained about 1000 oil bodies (Fig. 2A). The average diameter of these oil bodies was similar in (–)Su and (+)Su medium (Supplementary Table S1). Subsequently, the number of oil bodies progressively diminished in both culture variants but this process occurred more rapidly in pollen grains germinated in (–)Su medium. After 3 h of culture, oil bodies doubled their number and size in pollen tubes grown in (–)Su medium (Fig. 2B and Supplementary Fig. S2). The average number and size of oil bodies gradually decreased in both culture variants. However, lipid mobilization occurred more rapidly in pollen tubes growing in (–)Su medium and oil bodies were noticeably larger (Fig. 2B and Supplementary Table S1).

Detection of oil body-associated enzymatic activities in the olive pollen during germination

The oil body-associated protein profile is displayed in Fig. 3A. A single lipolytic band of 44.6 kDa was associated with oil bodies in the mature pollen, whereas a unique 43.1-kDa lipase was detected in the germinated pollen (Fig. 3B). A protein of about 99 kDa with LOX activity was also detected in oil bodies

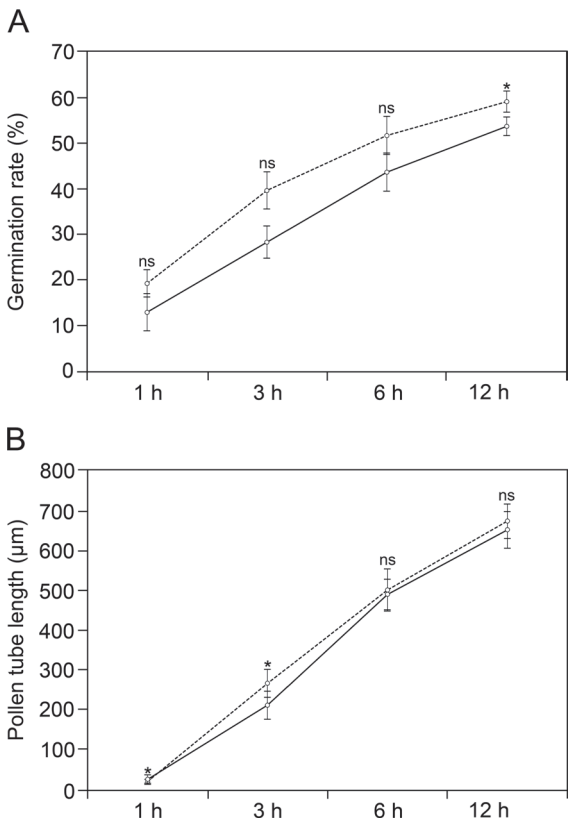

Fig. 1. Comparison of olive pollen germination rate (A) and pollen tube growth rate (B) in culture medium with sucrose (dashed line) and without sucrose (solid line). Values are mean ± SD calculated from 1000 (A) and 500 (B) pollen grains. Asterisks correspond to significant difference ($P \leq 0.01$); ns, no significant difference.

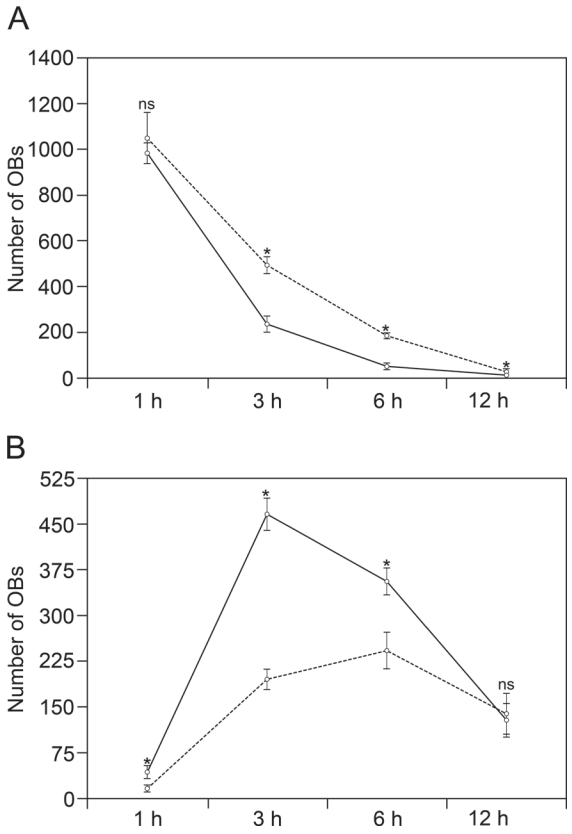

Fig. 2. Oil body dynamics in olive pollen grains (A) and pollen tubes (B) at different times of germination in culture medium with sucrose (dashed line) and without sucrose (solid line). Values are mean ± SD from Z-series images corresponding to 45 germinated pollen grains from three independent experiments ($N = 45$). Asterisks correspond to significant difference ($P \leq 0.01$); ns, no significant difference.

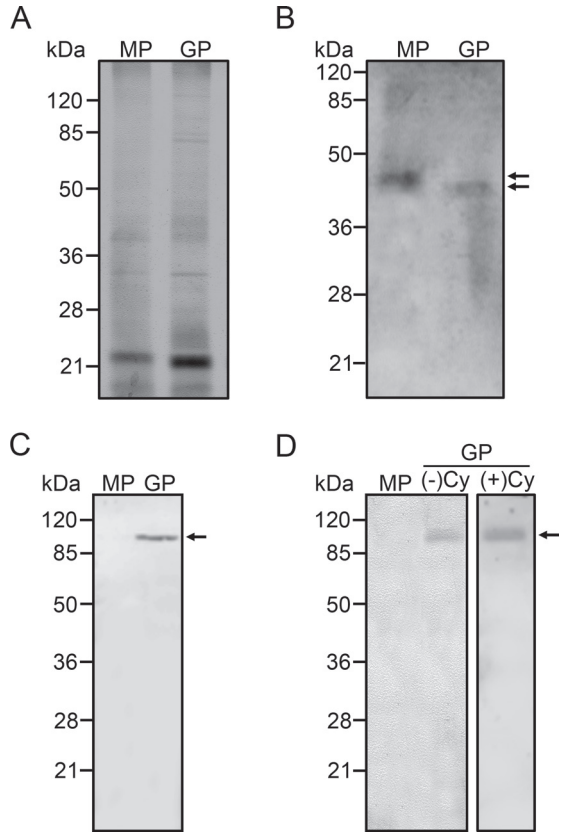

Fig. 3. (A) Coomassie-stained polyacrylamide gel showing mature (MP) and germinated (GP) pollen oil body-associated protein profiles. (B) *In-gel* assay of lipase activity (arrows). (C) Immunodetection of lipoxygenase (arrow). (D) *In-gel* assay of lipoxygenase activity (arrows) in experiments with (+Cy) or without (–Cy) cyanide.

of germinated pollen (Fig. 3C, D). The specificity of the anti-LOX antibody was confirmed by immunoprecipitation and MS/MS analyses (Supplementary Fig. S3 and Table S2).

Lipase activity values reached a peak after 3 h in both culture variants (Fig. 4A), but they were 2-fold higher in (–)Su samples. PLA activity profiles were similar to those of lipase (Fig. 4B) and PLA activity levels were also significantly higher in (–)Su samples. After 6 h of culture, the intensity of both lipase and PLA activities significantly decreased.

In-gel assays of enzyme activity revealed up to 12 putative lipases in germinated pollen in both (–)Su and (+)Su medium, with two prominent activity bands of 38.8 and 51.6 kDa (Fig. 5A). Lipase activities showed significantly higher intensities in (–)Su samples (Fig. 5A). Early steps of pollen germination were also accompanied by LOX activity, with bands of about 63, 90, and 99 kDa (Fig. 5B). Average levels of LOX activity were also higher in (–)Su samples (Fig. 5B).

Functional analysis of oil body-associated enzymes using specific inhibitors

When ebelactone B and ferulic acid inhibitors were added to the germination medium, they significantly hampered the capacity of pollen to germinate in the two culture variants assayed (Supplementary Fig. S4). This effect was accompanied by a characteristic accumulation of oil bodies in the germinative swollen aperture (Supplementary Fig. S5). Moreover, in-gel inhibition assays showed that LOX activities corresponding to 90- and 99-kDa bands were fully inhibited by the action of ferulic acid, while the 63-kDa band remained unaffected (Fig. 6).

Cellular localization of lipase and PLA activities in the olive pollen

Purified pollen oil bodies were visible as spherical structures that stained positively with Nile red (Supplementary Fig. S6). Isolated oil bodies did not show lipase activity at pollen maturity (Supplementary Fig. S7A). After 3 h of germination, oil bodies displayed a strong lipase activity on their surface (Supplementary Fig. S7B). This study could also detect the existence of PLA activity on the oil bodies boundaries in both mature and germinated pollen samples (Supplementary Fig. S7C, D).

The lipase activity was detected around the pollen wall and the vicinity of the pollen aperture from which the pollen tube emerged (Supplementary Fig. S8). In the pollen

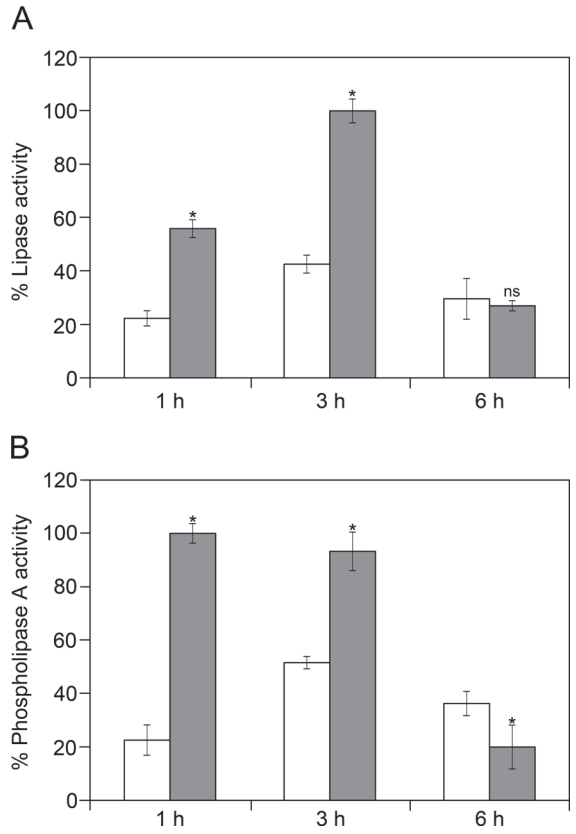

Fig. 4. *In vitro* assays of lipase activity (A) and phospholipase A activity (B) at different time intervals of olive pollen germination in culture medium with sucrose (white bars) and without sucrose (grey bars). Values are mean ± SD relative percentages referred to the highest optical density value for five independent experiments with three replicas each ($N = 15$) performed in each case. Asterisks correspond to significant difference ($P \leq 0.01$); ns, no significant difference.

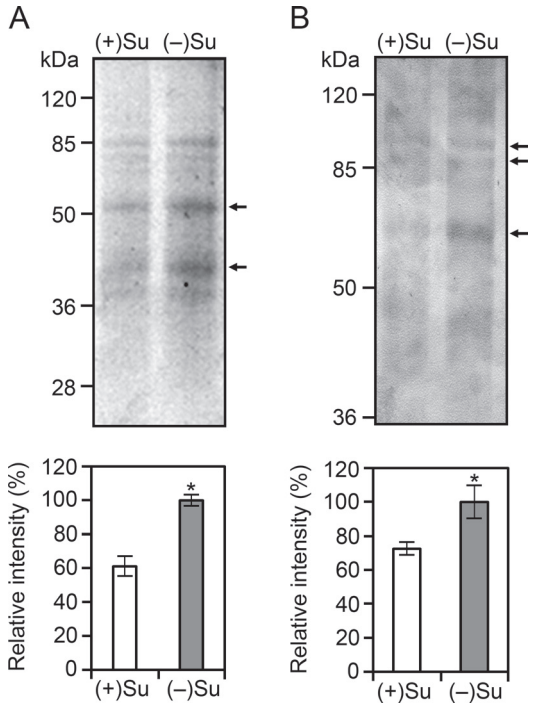

Fig. 5. (A) *In-gel* detection of lipase activity in 3-h germinated pollen in culture medium with sucrose [(+)Su and without sucrose [(–)Su]. Major activity bands are indicated with arrows. The chart at the bottom display the average densitometric data corresponding to the lipolytic bands detected on three gels. (B) *In-gel* detection of lipoxygenase activity in 3-h germinated pollen in (+)Su and (–)Su culture media. Major activity bands are indicated with arrows. The chart at the bottom displays the average densitometric data corresponding to the lipoxygenase bands detected on three gels. Asterisks correspond to significant difference ($P \leq 0.01$).

tube, the fluorescent signal was mainly located along the cytoplasm. The cytoplasmic labelling was visualized as red fluorescent spots (Supplementary Fig. S8, inset). The PLA activity was exclusively located in the pollen tube cytoplasm as green fluorescent spots (Fig. 7) and co-localized with oil bodies (Fig. 7, inset). Simultaneous fluorescent labelling with resorufin ester and BODIPY FL C_{11}-PC showed that lipase and PLA activities also co-localize in the pollen tube cytoplasm (Fig. 8). Negative controls did not show any fluorescence (Supplementary Fig. S9).

Immunolocalization of LOX enzyme in the olive pollen

Fluorescent immunolabelling of isolated oil bodies confirmed the presence of a LOX enzyme on the oil body surface (Supplementary Fig. S7E, F). In the incipient pollen tube, green fluorescence was found along the cytoplasm (Fig. 9A–D). After 3 h of culture, intense labelling, forming fluorescent spots and patches, was detected along the pollen tube cytoplasm except for the subapical region (Fig. 9E–H). Simultaneous labelling with Nile red showed some co-localization of LOX with oil bodies (Fig. 9H, insets). Green fluorescence was also visible at the pollen tube tip, where no oil bodies were detected. Negative controls did not show any fluorescence (Supplementary Fig. S9).

At ultrastructural level, the olive pollen LOX was mainly localized attached to the surface of oil bodies that appeared randomly distributed along the pollen tube cytoplasm (Fig. 10A). Moreover, a significant labelling was found attached to small cytoplasmic vesicles that fused with the plasma membrane, which was also decorated with gold particles (Fig. 10A). Negative controls showed no labelling (Fig. 10B).

Discussion

This work first addressed the question of whether olive pollen lipidic reserves are sufficient to promote the autonomous growth of the pollen tube. Sugar removal from the culture medium did not significantly influence pollen tube growth rate, suggesting that storage TAGs are sufficient as carbon supply for proper early pollen tube growth. Mobilization of seed storage lipids in *Arabidopsis thaliana* seedlings was

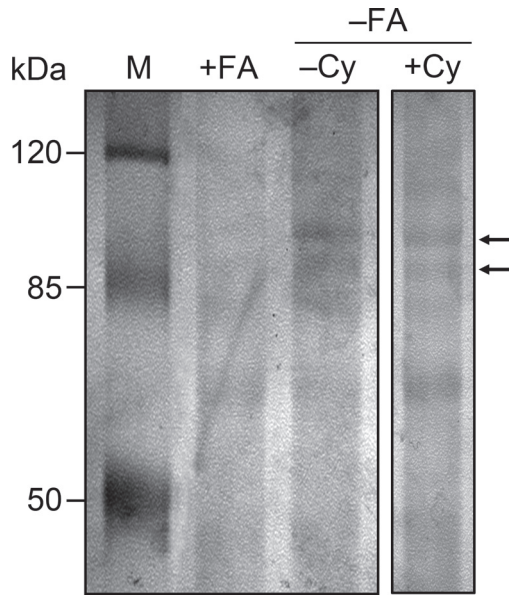

Fig. 6. *In-gel* inhibition assay of lipoxygenase activity in olive pollen after 3 h of *in vitro* germination. In experiments with 2 mM ferulic acid (+FA), lipoxygenase activities corresponding to 90 and 99 kDa bands (arrows) were inhibited. In control experiments (–FA), the lipoxygenase activity profile was similar with (+Cy) and without (–Cy) cyanide. M, protein markers.

significantly retarded in the presence of exogenous glucose (To *et al.*, 2002). This effect seems to be correlated with changes on expression of genes encoding lipid mobilization-related enzymes. Thus, lipase and catalase activities were significantly enhanced in yellow lupine seeds germinated in sugar-free medium (Borek *et al.*, 2006; Borek and Nuc, 2011). Similarly, the present study observed that oil body mobilization during olive pollen germination occurred more rapidly when the culture medium was not supplemented with sucrose. In parallel, under sugar-deprivation conditions, lipase, PLA, and LOX activities significantly increased in the olive pollen tube, confirming that the catabolism of storage lipids is more intense in the absence of an extracellular carbon supply.

The olive stigma exudate contains pectins and free sugars that are likely products of starch hydrolysis in papillae prior to pollination and may serve as pollen tube cell wall precursors (Suárez *et al.*, 2012). However, these extracellular sugars might also modulate the expression of oil body-associated enzymes during pollen tube growth on the stigma, as shown *in vitro*. Olive pollen tubes grow *in vitro* at rates of about 1 µm min^{-1} (this paper), and storage lipids are mostly degraded within 12 h of culture (Zienkiewicz *et al.*, 2010). Interestingly, oil bodies present in pollen and some arbuscular mycorrhizal fungi show a strong resemblance in terms of size and distribution and movement within pollen and germ tubes (Bago

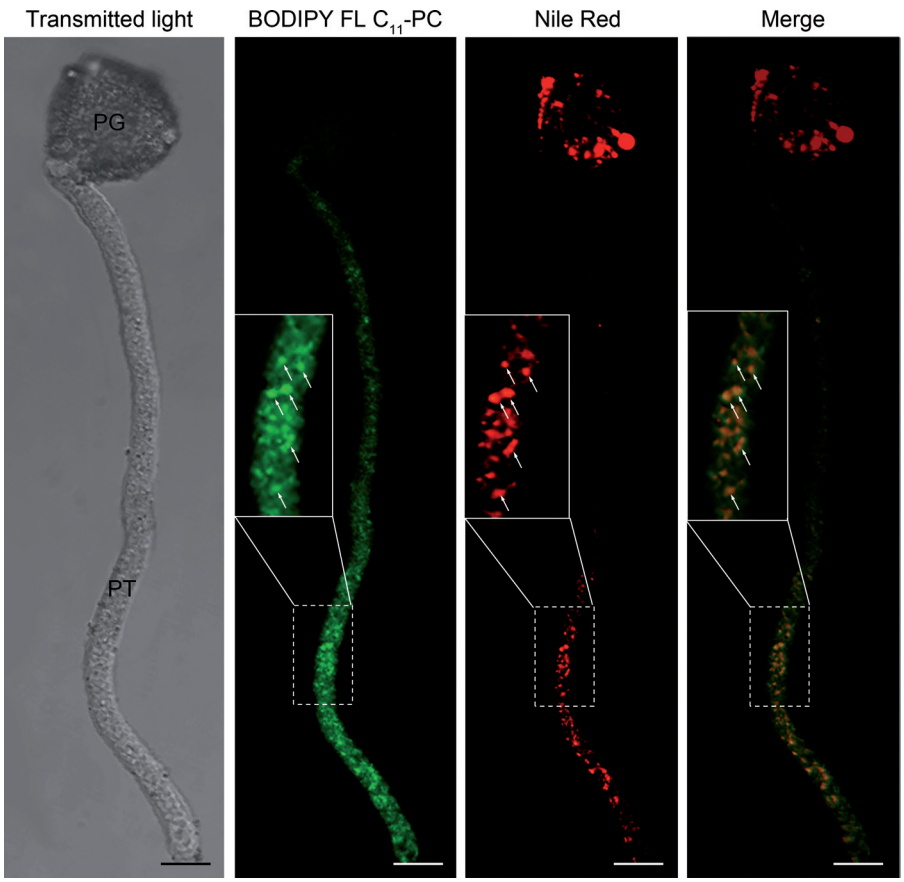

Fig. 7. Co-localization of phospholipase A activity (BODIPY FL C_{11} -PC) with oil bodies (Nile red) in olive pollen tubes (arrows) after 3 h of culture. Insets show the magnified areas marked with the dashed squares. PG, pollen grain; PT, pollen tube. Bars, 10 µm.

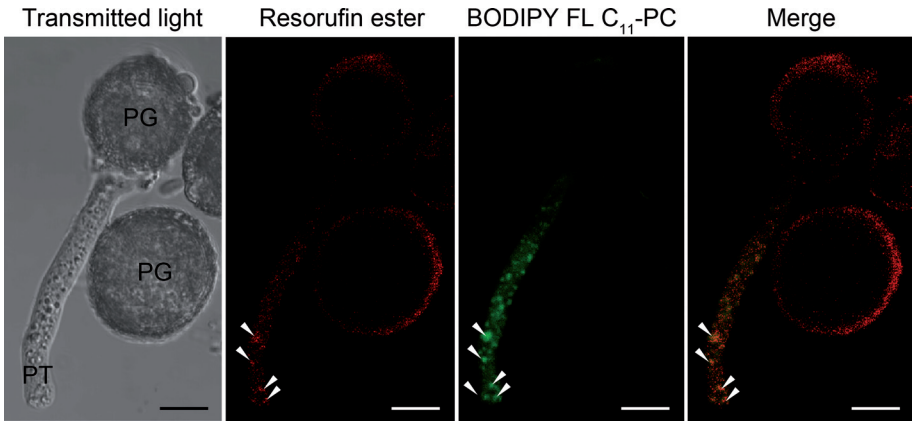

Fig. 8. Co-localization of lipase (resorufin ester) and phospholipase A (BODIPY FL C_{11}-PC) activities in olive pollen tubes (arrowheads) after 2 h of culture. PG, pollen grain; PT, pollen tube. Bars, 10 μm.

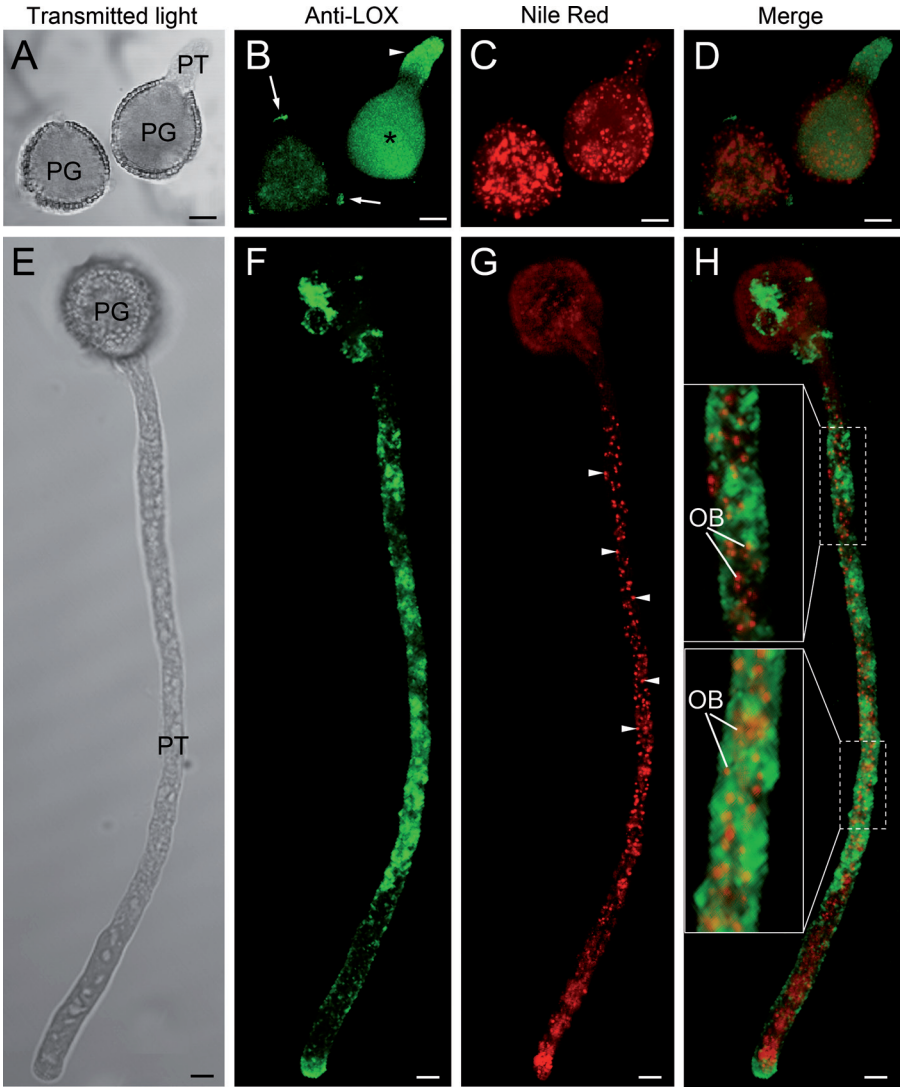

Fig. 9. Co-localization of lipoxygenase (anti-LOX antibody) with oil bodies (Nile red). (A–D) Optical section by confocal laser scanning microscopy of olive pollen cultured *in vitro* for 1 h. Fluorescent labelling of LOX was mainly located in the cytoplasm of the pollen grain (B, asterisk) and the pollen tube (B, arrowhead). In the non-germinated pollen grains, fluorescence was lower and located in the cytoplasm and the three apertures (B, arrows). (E–H) Optical section by confocal laser scanning microscopy of olive pollen cultured *in vitro* for 3 h. Fluorescent labelling of LOX was heterogeneously distributed along the pollen tube cytoplasm except for the subapical region. The pollen tube contained numerous oil bodies (G, arrowheads) that co-localized with LOX (H, insets). OB, oil body; PG, pollen grain; PT, pollen tube. Bars, 10 μm.

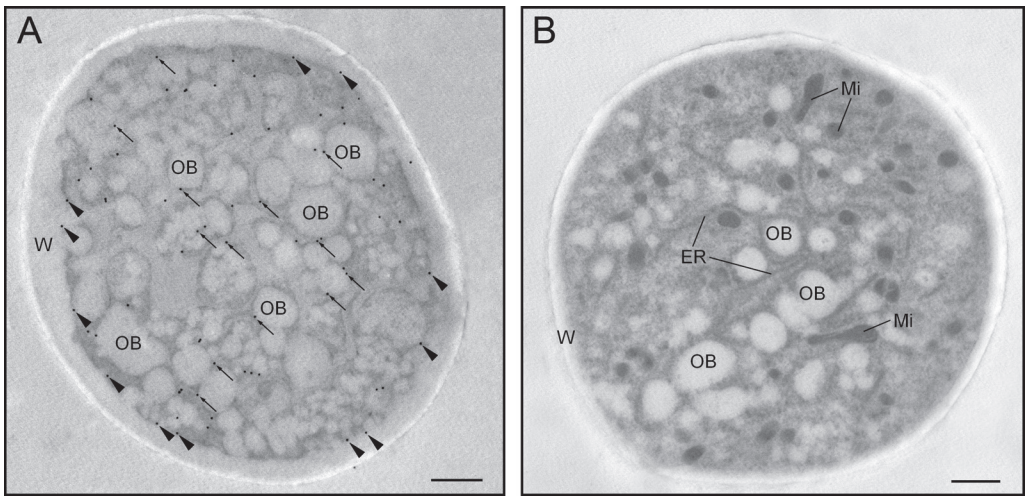

Fig. 10. Ultrastructural localization of lipoxygenase in the olive pollen tube. (A) Transversal section of a pollen tube after 3 h of germination. Gold particles were located on the oil bodies' boundaries (arrows) and in the surrounding cytoplasm. Some labelling was also present at the plasma membrane (arrowheads). (B) Negative control showing no gold labelling. ER, endoplasmic reticulum; Mi, mitochondria; OB, oil body; W, pollen tube wall. Bars, 1 μm.

et al., 2002). Pollen oil bodies are entirely mobilized in the olive stigma (unpublished data), and pollen tube growth is much faster than *in vitro* (Suárez *et al*., 2012). In the style and ovary, the passage of the pollen tubes across the transmitting track is accompanied by the disappearance of starch (Suárez *et al*. 2012). All these data suggest that the olive pollen tube growth in the stigma is mainly fuelled by its own reserves, but is markedly heterotrophic in the style and ovary.

Seed oil bodies are stabilized by proteins (e.g. oleosins) docked in the phospholipids that surrounds the TAG matrix (Huang, 1994). Therefore, a first step in oil body mobilization should be the disruption of this barrier in order to promote access of lipases to the stored TAGs (Noll *et al*., 2000; Gupta and Bhatla, 2007). A recent paper found evidence that the mobilization of storage lipids by LOX in cucumber cotyledons is promoted by a patatin-type phospholipase (Rudolph *et al*., 2011). Here, the present study has detected for the first time the presence of PLA activity associated to the surface of oil bodies. Moreover, a good temporal correlation was observed between PLA and lipase activities during germination. These data suggest that the action of the oil body-associated lipase might be promoted by the co-localizing PLA enzyme. Interestingly, the mature pollen grain also showed PLA activity suggesting that this enzyme may be active and stably associated to oil bodies at this developmental stage. This is in good agreement with the idea that phospholipids breakdown is a prerequisite for TAG mobilization.

The pollen coat lipids play a key role in pollen hydration on dry stigmas (Wolters-Arts *et al*., 1998). Lipases are present in the extracellular pollen coat that is deposited on the pollen wall surface at the final steps of pollen maturation (Mayfield *et al*., 2001; Shakya and Bhatla 2010). Recently, it has been demonstrated that the extracellular lipase *EXL4* is required for efficient hydration of *Arabidopsis* pollen (Updegraff *et al*., 2009). Similarly, the olive pollen wall surface showed a strong lipase activity. Whether such enzyme activity has a function in olive pollen hydration is still a matter for future investigations.

The lipase activity was also tightly associated to the surface of olive pollen oil bodies. As far as is known, this is the first study reporting the intracellular localization of lipases in the pollen grain. It is widely accepted that storage lipid breakdown during oilseed germination is initiated by the activation of TAG lipases, which hydrolyse TAGs and release glycerol and fatty acids (Graham, 2008). In a previous study, Rejón *et al*. (2012) examined the lipolytic capacity of the mature olive pollen and identified up to 12 putative lipases. Here, the present study has identified a unique lipolytic band associated to olive pollen oil bodies. Interestingly, the lipolytic activity was only detectable on oil bodies in germinated pollen grains. In the light of the biochemical and microscopy findings reported here, this lipase enzyme might be inhibited *in vivo* at the mature pollen stage, being further activated during germination. Indeed, some studies carried out in seeds suggested that lipase activity could be inhibited by acyl-CoAs (plus CoA) (Hills *et al*., 1989). In a previous work, it was demonstrated that olive pollen lipase activity is effectively inhibited both *in vitro* and *in gel* by ebelactone B (Rejón *et al*., 2012). Here, the present study found that the addition of ebelactone B to the culture medium resulted in strong reduction of germination capacity of olive pollen, likely by hampering storage lipid mobilization. The size of the olive pollen oil body-associated lipase is similar to LeLID1 (45 kDa) and AtLIP1 (42 kDa) seed TAG lipases from tomato and *Arabidopsis*, respectively. The gene *SDP1* encodes a 95-kDa patatin domain TAG lipase that is involved in storage lipid mobilization in germinating *Arabidopsis* seeds (Eastmond 2006). LeLID1 and AtLIP1, however, do not possess the patatin domain and they are not directly involved in oil body breakdown (Matsui *et al*., 2004; El-Kouhen *et al*., 2005). At present, there is a lack of conclusive evidence that a TAG lipase is involved in storage lipid mobilization in olive

pollen. Further analysis will be necessary to determine if the pollen oil body-associated lipase is a bona-fide TAG lipase.

Fatty acids released from TAGs may be further degraded either via the β-oxidation in glyoxysomes (Graham, 2008), or by an alternative pathway, which is dependent on a LOX enzyme (Feussner *et al*., 1994). The present study detected for the first time the existence of LOX activity in germinating olive pollen. The anti-LOX antibody used in this work recognized a unique 99-kDa protein band on Western blots. However, the LOX activity profile revealed the existence of a second LOX isoenzyme with an estimated molecular weight of ~90 kDa. The size of both olive pollen LOXs are in the molecular weight range reported for other plant LOXs (Andreou and Feussner, 2009). The present study also detected a smaller protein band of ~63 kDa, which showed enhanced LOX activity. Interestingly, limited proteolysis of soybean LOX1 generated a ~60-kDa fragment with enhanced activity and membrane-binding ability (Maccarrone *et al*., 2001). Therefore, it is plausible that the 63-kDa LOX found in the olive pollen comes from one of the other two larger LOXs, although this point needs to be experimentally verified.

The olive pollen 99-kDa LOX was mainly localized on the oil body surface, as previously reported for other LOX enzymes in seeds and cotyledons (Rodríguez-Rosales *et al*., 1998; Rudolph *et al*., 2011; Yadav and Bhatla, 2011), which suggests that it is a type 1-LOX. The present study could not detect either the protein or its activity in oil bodies from mature pollen grains. This fact suggests that the 99-kDa LOX enzyme might be recruited by oil bodies just after pollen hydration. Olive pollen LOX in-gel activity was effectively inhibited by ferulic acid, as previously demonstrated for other plant LOXs (Redrejo-Rodríguez *et al*., 2004; Szymanowska *et al*., 2009). The present study found that the capacity of olive pollen to germinate was seriously hampered when ferulic acid was added to the culture medium. This effect was characteristically accompanied by a strong accumulation of oil bodies at the germinative aperture. However, there is no conclusive experimental evidence that ferulic acid only inhibits LOX activity, so these results should be taken cautiously. Further functional analysis will be necessary to confirm that pollen performance is affected when LOX enzyme is blocked. All together, these data support the idea that a LOX-mediated pathway of storage lipid mobilization, which is of major importance for germination, exists in the olive pollen grain.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. *In vitro* germination rates of olive pollen in sucrose-supplemented and sucrose-free medium.

Supplementary Fig. S2. Nile red staining of oil bodies in olive pollen germinated in sucrose-supplemented and sucrose-free medium.

Supplementary Fig. S3. Immunoprecipitation of olive pollen lipoxygenase.

Supplementary Fig. S4. Effect of lipase and lipoxygenase inhibitors on olive pollen germination.

Supplementary Fig. S5. Inhibitory effects of ferulic acid and ebelactone B on pollen germination.

Supplementary Fig. S6. Nile red staining of olive pollen oil bodies.

Supplementary Fig. S7. Detection of lipase, phospholipase A, and lipoxygenase on the oil body surface.

Supplementary Fig. S8. Immunolocalization of lipase activity in olive pollen during germination.

Supplementary Fig. S9. Negative controls for lipase, phospholipase A, and lipoxygenase detection.

Supplementary Table S1. Average size of olive pollen oil bodies during *in vitro* germination.

Supplementary Table S2. Identification of olive pollen lipoxygenase.

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