

RESEARCH PAPER

Identification and localization of a caleosin in olive (*Olea europaea* L.) pollen during *in vitro* germination

Krzysztof Zienkiewicz^{1,2,†}, Antonio J. Castro^{1,†}, Juan de Dios Alché¹, Agnieszka Zienkiewicz^{1,3}, Cynthia Suárez¹ and María Isabel Rodríguez-García^{1,*}

¹ Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín (CSIC), Profesor Albareda 1, 18008, Granada, Spain

² Department of Cell Biology, Institute of General and Molecular Biology, Nicolaus Copernicus University, Gargarina 9, 87-100, Toruń, Poland

³ Department of Physiology and Molecular Biology of Plants, Institute of General and Molecular Biology, Nicolaus Copernicus University, Gargarina 9, 87-100, Toruń, Poland

[†] These authors contributed equally to this work.

* To whom correspondence should be addressed. E-mail: mariaisabel.rodriguez@eez.csic.es

Received 20 October 2009; Revised 23 December 2009; Accepted 21 January 2010

Abstract

In plant organs and tissues, the neutral storage lipids are confined to discrete spherical organelles called oil bodies. Oil bodies from plant seeds contain 0.6–3% proteins, including oleosins, steroleosins, and caleosins. In this study, a caleosin isoform of ~30 kDa was identified in the olive pollen grain. The protein was mainly located at the boundaries of the oil bodies in the cytoplasm of the pollen grain and the pollen tube. In addition, caleosins were also visualized in the cytoplasm at the subapical zone, as well as in the tonoplast of vacuoles present in the pollen tube cytoplasm. The cellular behaviour of lipid bodies in the olive pollen was also monitored during *in vitro* germination. The number of oil bodies decreased 20-fold in the pollen grain during germination, whereas the opposite tendency occurred in the pollen tube, suggesting that oil bodies moved from one to the other. The data suggest that this pollen caleosin might have a role in the mobilization of oil bodies as well as in the reorganization of membrane compartments during pollen *in vitro* germination.

Key words: Caleosin, *in vitro* germination, oil bodies, *Olea europaea*, olive, pollen grain, pollen tube.

Introduction

Eukaryotes contain large amounts of neutral lipids (Murphy, 2001). In plant organs and tissues (e.g. seeds), the neutral storage lipids are confined to discrete spherical organelles of ~0.5–2 µm (Huang, 1996) called oil bodies (OBs). They have been proposed to consist of a core containing neutral lipids [triacylglycerols (TAGs), sterol esters, etc.] surrounded by a single layer of phospholipids with a few embedded unique proteins (Tzen *et al.*, 1993; Huang, 1996). OBs represent the primary energy reserve to support periods of active metabolism (Murphy and Vance, 1999). However, despite OBs having often been regarded as

simple storage sites, a plethora of unsuspected dynamic roles has been suggested for these organelles, including subcellular lipid trafficking and turnover, and calcium signalling (Frandsen *et al.*, 2001; Poxleitner *et al.*, 2006).

OBs from plant seeds contain 0.6–3% proteins (Tzen *et al.*, 1993). Oleosins are by far the most abundant OB-associated proteins (Huang, 1996). Two additional classes of proteins, namely caleosins and steroleosins, have been identified in OBs from seeds (Frandsen *et al.*, 1996; Chen *et al.*, 1999; Lin *et al.*, 2002; Lin and Tzen, 2004). Caleosins belong to a large gene family found ubiquitously

Abbreviations: Ab, antibody; BSA, bovine serum albumin; DTT, dithiothreitol; EDTV, electron-dense thin vesicle; ER, endoplasmic reticulum; MALDI, matrix-assisted laser desorption/ionization; OB, oil body; PBS, phosphate-buffered saline; PVPP, polyvinylpyrrolidone; TAG, triacylglycerol; TBS, TRIS-buffered saline; TCA, trichloroacetic acid; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; TOF, time of flight.

© 2010 The Author(s).

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/2.5>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Downloaded from <http://jxb.oxfordjournals.org> by on 18 February 2010

in higher plants but also in several lipid-accumulating fungi such as *Neurospora crassa* (accession number AI329048) and *Aspergillus nidulans* (AA787643), and the single-celled algae *Chlorella protothecoides* (AJ238627). All caleosins contain a calcium-binding domain consisting of a conserved EF-hand capable of binding a single calcium atom (Frandsen *et al.*, 1996), a central hydrophobic region with a potential lipid-binding domain, and a C-terminal region including several putative protein kinase phosphorylation sites. Caleosins have been located either on the surface of OBs or associated with an endoplasmic reticulum (ER) subdomain (Næsted *et al.*, 2000). Recent works suggest that these proteins might be involved in signal transduction via calcium binding or phosphorylation/dephosphorylation in processes such as membrane expansion, lipid trafficking, and lipid body biogenesis and degradation (Frandsen *et al.*, 2001; Poxleitner *et al.*, 2006). A recent study showed a caleosin that possess peroxxygenase activity, suggesting a role in phytooxylipin metabolism as well (Hanano *et al.*, 2006).

Olive (*Olea europaea* L.) is a typical oil-storing plant species. Several olive organs and tissues have been reported to contain large amounts of OBs (Pacini and Juniper, 1979; Ross *et al.*, 1993; Alché *et al.*, 1999a, 2006; Rodríguez-García *et al.*, 2003). The olive pollen grain accumulates a high number of OBs at maturity, which spread over the cytoplasm of the vegetative cell. During pollen hydration, these organelles polarize near the aperture through which the pollen tube emerges (Rodríguez-García *et al.*, 2003). This fact suggests that OBs might be involved in olive pollen germination and early pollen tube growth in the pistil. However, nothing is known about the behaviour of OBs during such a process. Recently, a unique caleosin isoform distinct from that in seed OBs has been identified in lily pollen (Jiang *et al.*, 2008). Nevertheless, no research data regarding the presence and function of caleosins during pollen germination and pollen tube growth have been published to date.

The present paper reports the identification of a new OB-associated caleosin in olive (*O. europaea* L.) pollen and its cellular location during *in vitro* pollen germination. The behaviour of OBs during *in vitro* pollen germination was also studied. Finally, the possible involvement of this caleosin in the mobilization of OBs and the reorganization of membrane compartments during this process is discussed.

Materials and methods

Pollen material and sampling

Olive (*O. europaea* L. cv. Picual) mature pollen grains were harvested from dehiscent anthers by vigorous shaking of flowering shoots inside large paper bags. Sampling was carried out from discrete trees of the collection of olive varieties of the CIFA 'Venta del Llano' (Jaén, Spain). Pollen samples were sieved through an appropriate set of meshes to remove floral debris and processed fresh or stored at -80°C until use.

In vitro germination of olive pollen

Pollen was pre-hydrated by incubation in a humid chamber at room temperature for 30 min and then transferred to Petri dishes

(0.1 g per dish) containing 10 ml of germination medium [10% (w/v) sucrose, 0.03% (w/v) $\text{Ca}(\text{NO}_3)_2$, 0.01% (w/v) KNO_3 , 0.02% (w/v) MgSO_4 , and 0.01% (w/v) boric acid]. Petri dishes were maintained at room temperature in the dark, and pollen grains were sampled 1, 2, 3, 6, and 12 h after the onset of the *in vitro* germination.

Purification of OBs and the microsomal fraction from olive pollen

The isolation of OBs and the microsomal fraction from olive pollen tubes was carried out as described by Hernández-Pinzón *et al.* (2001). All steps were performed at 4°C . Samples were ground in a homogenization buffer consisting of 100 mM HEPES buffer (pH 7.5) containing 0.4 M sucrose, 10 mM KCl, 1 mM EDTA, and 2 mM dithiothreitol (DTT). Homogenates were centrifuged at 6000 g for 2 min to remove debris. They were then fractionated by centrifugation at 20 000 g for 20 min. The lower supernatant was further centrifuged at 100 000 g for 1 h. The pellet, corresponding to the microsomal fraction, was recovered and then resuspended in homogenization buffer and stored at -20°C until use. The upper lipid pad was resuspended in 50 mM TRIS-HCl buffer (pH 7.2) containing 1 M NaCl, 9 M urea, and 0.2% (w/v) Tween-20. The suspension was diluted with 0.5 ml of homogenization buffer and layered with 4 vols of 0.1 M sucrose in 100 mM HEPES buffer (pH 7.5). OBs were recovered after centrifugation as above. Washing steps were performed according to Jiang *et al.* (2007). OBs were washed in an equal volume of 0.1% (v/v) Nonidet P40 (NP40) substitute (Sigma-Aldrich, St Louis, MO, USA) and 0.2 M sucrose in 5 mM sodium phosphate buffer, pH 7.5. They were then centrifuged at 15 700 g for 30 min and the upper fraction was collected and mixed with an equal volume of 2 M NaCl and 0.6 M sucrose in 10 mM sodium phosphate buffer (pH 7.5). Finally, OBs were recovered upon centrifugation as above, mixed with 0.6 M sucrose in 10 mM sodium phosphate buffer (pH 7.5), and stored at -20°C until use.

Staining of OBs

After *in vitro* germination, pollen samples were fixed in 4% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) at 4°C overnight. After three washes in cacodylate buffer for 10 min each, pollen samples were resuspended in an anti-fading solution of Citifluor (Sigma-Aldrich). Aliquots (50 μl) of germinated pollen were mixed with 10 μl of a solution of 0.1 mg ml^{-1} Nile Red (Sigma-Aldrich) in acetone. Samples were observed with a C1 confocal laser scanning microscope (Nikon, Japan) using an argon (488 nm) laser. Z-series images were collected and processed using the EZ-C1 Gold v.2.10 build 240 software (Nikon).

Aliquots (25 μl) of purified pollen OBs were incubated either with a solution of Sudan Black B 70% (w/v) in ethanol at 60°C for 1 min or with a solution of Nile Red as above. Samples were observed with an Axioplan epifluorescence microscope (Carl Zeiss, Germany). Digital images were recorded with a ProgRes C3 camera (Jenoptik Laser, Germany) using the ProgRes CapturePro software (Jenoptik Laser).

OB morphometry

Measurements of the number and size (the largest diameter) of OBs from pollen grains and pollen tubes after hydration and at different germination times (1, 3, 6, and 12 h) were carried out from digital images recorded with a C1 confocal laser scanning microscope (Nikon) and processed using the software EZ-C1 Gold version 2.10 build 240 (Nikon). Z-series images of five germinated pollen grains from three independent experiments ($n=15$) were captured and used for measurements and statistical analysis. The mean and the standard deviation were calculated and plotted using the SigmaPlot software (Systat Software, Germany).

Analysis of OB dynamics in living pollen tubes

Pollen samples were germinated *in vitro* for 3 h as described above. A 25 μ l aliquot of the germination medium was transferred onto a cover slide and mixed with 0.5 μ l of a solution containing 0.05 mg ml⁻¹ (w/v) Nile Red (Sigma-Aldrich) in 50% (v/v) acetone. Then, samples were immediately analysed with a C1 confocal laser scanning microscope (Nikon). Time-lapse capture was performed using the EZ-C1 Gold v.2.10 build 240 software (Nikon).

Protein extraction

Both mature and germinated pollen samples (0.1 g) were resuspended in 1.5 ml of extraction buffer A [40 mM TRIS-HCl, pH 7.4, 2% (v/v) Triton X-100, 1 mg ml⁻¹ ascorbic acid, 60 mM DTT, 5% (w/v) polyvinylpyrrolidone (PVPP), and 15 μ l of a protease inhibitor cocktail (Sigma-Aldrich)] and stirred for 6 h at 4 °C. After centrifugation at 10 000 g for 30 min at 4 °C, the supernatants containing the eluted proteins were transferred to 15 ml tubes and mixed with 10 vols of a solution consisting of 20% (w/v) trichloroacetic acid (TCA) and 0.2% (w/v) DTT in acetone. Proteins were precipitated at -20 °C overnight. Samples were then centrifuged at 10 000 g for 30 min at 4 °C. The resulting pellets were rinsed with acetone three times for 15 min each, resuspended in Laemmli (1970) sample buffer, dispensed into aliquots, and stored at -20 °C.

Isolated OBs were mixed with extraction buffer B [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 3% (w/v) SDS, 60 mM DTT, 0.5% (v/v) Bio-Lyte 3-10 buffer (Bio-Rad, Hercules, CA, USA), and traces of bromophenol blue] to a proportion of 1:2 and incubated for 1 h at 4 °C with occasional vortexing. The mixture was transferred to 15 ml tubes and processed as above.

The protein concentration in each sample was estimated by using the 2D Quant Kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

SDS-PAGE and western blotting

SDS-PAGE was performed according to Laemmli (1970). A 25 μ g aliquot of proteins per sample was loaded on 12% (w/v) polyacrylamide gels with 4% stacking gels using a Mini-Protein 3 apparatus (Bio-Rad). After electrophoresis, gels were stained with Coomassie Brilliant Blue R250 according to standard procedures. Proteins were electroblotted onto a PVDF membrane in a Semi-Dry Transfer Cell (Bio-Rad). The membrane was blocked overnight at 4 °C in a solution containing 3% (w/v) bovine serum albumin (BSA) in TRIS-buffered saline (TBS) buffer, pH 7.4. Immunodetection of caleosins was carried out by incubation with a polyclonal antibody (Ab) prepared from the complete sequence of Clo3 caleosin from *Arabidopsis thaliana*, diluted 1:500 in TBS buffer (pH 7.4) containing 0.3% (v/v) Tween-20, for 12 h at 4 °C. An Alexa Fluor 633-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR, USA), diluted 1:2000, served as the secondary Ab. The signal was detected in a Pharos FX molecular imager (Bio-Rad). The specificity of the anti-Clo3 Ab was tested by omitting this Ab from the protocol.

Caleosin immunoprecipitation

Total proteins were extracted from pollen and isolated OBs as described above. After TCA precipitation, pellets were resuspended in 200 μ l of a TBS solution containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.5% (v/v) NP40, 1% (v/v) Triton X-100, and 10 μ l (0.6 μ g) of the anti-Clo3 Ab. Samples were incubated for 4 h at 4 °C under gentle agitation, and 30 μ l of protein A-Sepharose were added. Samples were then incubated for 2 h at 4 °C under gentle agitation, and immunoprecipitated complexes were collected by centrifugation at 20 000 g for 15 min at 4 °C. The resulting pellets were washed three times with 0.5 ml of TBS buffer containing 1% (v/v) Triton X-100 and 0.5% (v/v) NP40, and resuspended in 50 μ l of Laemmli sample buffer.

SDS-PAGE and western blotting were conducted as described above. After electrophoresis, gels were stained with Coomassie Brilliant Blue R250 according to standard procedures. The specificity of the anti-Clo3 Ab was tested by omitting the primary Ab from the protocol.

Caleosin identification by mass spectrometry

The 34 kDa and 29 kDa protein bands were excised from Coomassie-stained gels in an EXQuest Spot Cutter (Bio-Rad). Proteins were reduced by 10 mM DTT at 56 °C for 45 min and then alkylated by 55 mM iodoacetamide at room temperature for 30 min. Proteins were then subjected to in-gel digestion with trypsin (Trypsin Gold, Promega, Madison, WI, USA) at 37 °C for 4 h in a Digest Pro MS station (Intavis, Germany). The resulting peptides were purified using a ZipTip μ -C18 column (Millipore, Bedford, MA, USA), and eluted with 0.2% (v/v) trifluoroacetic acid (TFA). Samples (1 μ l) were co-crystallized with a matrix solution of 2.5 mg ml⁻¹ α -cyano-4-hydroxycinnamic acid (1 μ l) onto MALDI (matrix-assisted laser desorption/ionization) chips. Finally, samples were subjected to MALDI-time of flight (TOF) analysis in a Voyager DE-PRO spectrometer (Applied Biosystems, Foster City, CA, USA) in positive reflection mode. An internal calibration was performed using the *m/z* of peptides resulting from the autolysis of trypsin.

The MS spectra were used to search algorithms against the NCBI and Swiss-Prot databases using a local Mascot™ (Matrix Science Ltd, UK) server. To be accepted for the identification, an error of <100 ppm on the parent ion mass was tolerated. One missed cleavage per peptide was allowed, and carbamidomethylation for cysteine and oxidation for methionine were also considered.

Immunolocalization of caleosins in isolated OBs

Purified OBs were incubated in a 1.5 ml tube with the anti-Clo3 Ab (diluted 1:30 in grinding medium) for 2 h at room temperature, followed by incubation with an anti-rabbit IgG Ab conjugated with Alexa Fluor 488 (Molecular Probes), diluted 1:200 in grinding medium, for 1 h at 37 °C under gentle agitation. A few drops of an anti-fading solution of Citifluor (Sigma-Aldrich) were added and samples were observed with a Zeiss Axioplan epifluorescence microscope under blue light irradiation. Negative controls were treated as above, but the primary Ab was omitted.

Whole-cell immunodetection of caleosins

Mature and germinated pollen samples were fixed in a mixture of 4% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde prepared in 0.2 M sodium cacodylate buffer (pH 7.2) at 4 °C overnight. After two washes in cacodylate buffer, pollen tube cell walls were digested using a mixture of 1% (w/v) cellulase R10 (Serva, Germany) and 27 U of pectinase (Sigma-Aldrich) per ml in 0.01 M citrate buffer (pH 4.8) for 25 min at 37 °C.

After blocking with a solution containing 1% (w/v) BSA in phosphate-buffered saline (PBS) buffer (pH 7.2) for 1 h, samples were incubated with the anti-Clo3 primary Ab [diluted 1:50 in PBS buffer, pH 7.2, containing 1% (w/v) BSA] overnight at 4 °C. An anti-rabbit IgG Ab conjugated with Alexa Fluor 633 (Molecular Probes) diluted 1:200 in PBS buffer (pH 7.2) was used as the secondary Ab. Finally, samples were re-suspended in an anti-fading solution of Citifluor (Sigma-Aldrich). Just before examination, 10 μ l of Nile Red solution (prepared as described above) were added to each sample. Samples were then sequentially observed with a Nikon C1 confocal laser scanning microscope (Nikon, Japan) using a He-Ne (633 nm) laser for Alexa Fluor 633 detection and an argon (488 nm) laser for Nile red staining visualization. Z-series images were collected and processed with the software EZ-C1 Gold version 2.10 build 240 (Nikon). Negative controls were treated as above, but the primary Ab was omitted.

Immunolocalization of caleosins by TEM

Samples were fixed overnight in ice-cold 0.1 M cacodylate buffer, pH 7.2, containing 4% (v/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde. They were then washed in the same buffer, dehydrated in a graded ethanol series, and embedded in Unicryl resin (BBInternational, UK). Ultrathin sections (70 nm thick) were cut on an Ultracut microtome (Reichert-Jung, Germany) and mounted on 200 mesh formvar-coated nickel grids.

Blocking of non-specific binding sites was carried out by incubation of the sections in a solution containing 1% (w/v) BSA in PBS buffer, pH 7.2, for 1 h. This was followed by treatment with the anti-Clo3 Ab (diluted 1:50 in blocking solution) overnight at 4 °C. Samples were then rinsed with PBS buffer and incubated with an anti-rabbit IgG Ab conjugated with 15 nm gold particles (BBInternational) diluted 1:100 in 0.5% (w/v) BSA in PBS buffer, pH 7.2, for 1 h at room temperature. After washing, sections were stained with 5% (w/v) uranyl acetate for 30 min at room temperature. Observations were carried out with a JEM-1011 transmission electron microscope (JEOL, Japan). Control reactions were carried out by omitting the primary Ab.

Results

Identification of a caleosin from olive pollen

Immunoblot experiments showed the presence of a polypeptide of ~30 kDa, which was cross-recognized by the anti-Clo3 Ab (Fig. 1). Although this polypeptide was detected in all stages analysed, densitometric data showed the presence of quantitative differences. The highest levels of caleosin were observed immediately after hydration. At the early steps of pollen germination, levels slightly declined but they rapidly recovered after 2 h of germination. From this stage onwards, caleosin levels progressively decreased, to almost completely disappear after 12 h of *in vitro* germination.

Immunoprecipitation experiments showed that, when incubated with total protein extracts from mature pollen, the anti-Clo3 Ab bound to one protein of 30 kDa (Fig. 2). This band was also present after incubating the Clo-3 Ab with total proteins isolated from both OBs and the microsomal fraction. MS analyses of the tryptic peptides resulting from the digestion of the 30 kDa protein band matched two peptides of a caleosin (accession number EF015588) from lily pollen (Table 1).

OB behaviour during *in vitro* pollen germination

Neutral lipids were detected in both mature and germinating pollen grains as intensively fluorescent round-shaped structures in the cytoplasm (Fig. 3). The diameter of these OBs ranged from 0.2 µm to 2.1 µm. After hydration, ~1000±200 OBs per pollen grain were counted. An intense staining of the pollen wall surface was also observed (Fig. 3A). Neither the generative cell nor the vegetative nucleus showed any fluorescent labelling. OBs were also present in all the *in vitro* germination stages (Fig. 3B–F), but they showed significant differences with regard to number and size. Thus, in the cytoplasm of the pollen grain, the number of OBs decreased to <20±7.8 OBs after 12 h of germination (Fig. 4A). In contrast, the average number of

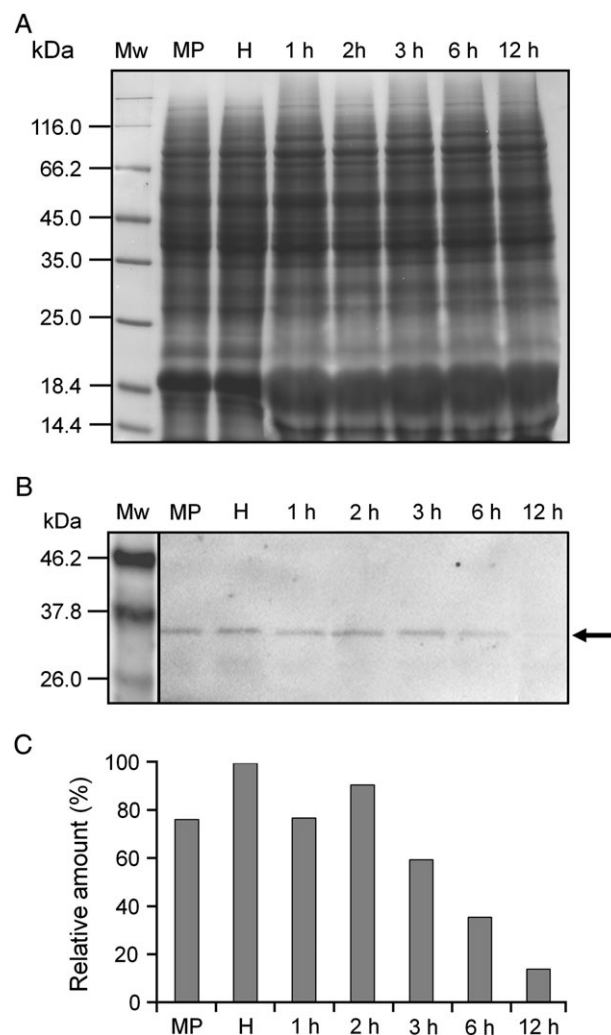


Fig. 1. Expression analysis of olive pollen caleosins. (A) Coomassie-stained gel of total proteins from olive pollen at maturity (MP), after hydration (H), and after 1, 2, 3, 6, and 12 h of *in vitro* germination. (B) Immunoblot as in A probed with the anti-Clo3 Ab. (C) Densitometric data corresponding to the 30 kDa band from B.

OBs significantly increased in the cytoplasm of the pollen tube during the first 3 h of germination (Fig. 4A), reaching a maximum of 248 ± 68.3 OBs per pollen tube. At later stages, the average number of OBs progressively decreased in the pollen tube cytoplasm. In parallel, the average size (i.e. diameter) of OBs progressively diminished during *in vitro* germination (Fig. 4B). Live-cell imaging of olive pollen tubes in combination with Nile red staining of OB-associated lipids allowed study of the dynamics of OB motility. During early steps of *in vitro* germination, OBs displayed intense dynamics, moving all along the pollen tube length following cytoplasmic streaming. This movement occurred in two ways, both from the pollen grain to the subapical region of the pollen tube and backwards. The growing pollen tube tip was not affected by this OB movement (Supplementary Video S1 available at JXB online). Once the pollen tubes grow, the movement of the OBs was initially slowed down by the presence of vacuoles, and finally restricted to the cytoplasmic regions distal from

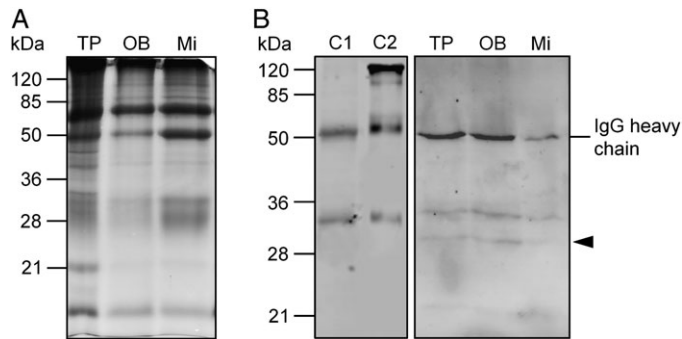


Fig. 2. (A) Anti-Clo3 Ab-immunoprecipitated proteins after SDS-PAGE and Coomassie staining. (B) Immunoblot as in A probed with the anti-Clo3 Ab. C1, control 1, anti-caleosin Ab probed with a secondary Ab; C2, control 2, anti-Clo3 Ab-immunoprecipitated fraction from pollen OB-associated proteins probed as in C1; TP, anti-Clo3 Ab-immunoprecipitated fraction from crude protein extracts from pollen; OB, anti-Clo3 Ab-immunoprecipitated fraction from pollen OB-associated proteins; Mi, anti-Clo3 Ab-immunoprecipitated fraction from the pollen microsomal fraction-associated proteins. The arrowhead shows the 30 kDa caleosin band.

Table 1. Caleosin peptides from olive pollen identified by MALDI-TOF/MS analysis.

Start–end	<i>M_r</i> (obs)	<i>M_r</i> (exp)	ΔM_r	Miss	Peptide sequence
214–227	1598.8100	1598.7664	0.0436	1	ETIRGAFDGSLFER
218–227	1098.5615	1098.5214	0.0401	0	GAFDGSLFER

the pollen grain by the existence of callose plugs (Supplementary Video S2).

Immunolocalization of caleosins in isolated OBs

After purification, olive pollen OBs were visible as spherical structures with a diameter varying from 0.2 μm to 2 μm (Fig. 5A, B). Their lipidic nature was confirmed by Sudan Black B and Nile red staining. Immunolocalization experiments with anti-Clo3 Ab allowed confirmation of the presence of caleosins in these organelles (Fig. 5C, D). Caleosin labelling was found on the surface of OBs, often being visualized as ring-shaped fluorescent structures.

Immunolocalization of caleosins in the olive pollen during *in vitro* germination

In the mature pollen grain sections, caleosins were located in the cytoplasm, the exine, and on the pollen coat, but not in the intine (Supplementary Fig. S1A at JXB online). The control did not show any fluorescent labelling (Supplementary Fig. S1B).

Whole-mount OB staining and caleosin immunodetection were carried out in parallel in both hydrated and germinated pollen grains. In the hydrated pollen, the signal corresponding to caleosins and OBs co-localized in the

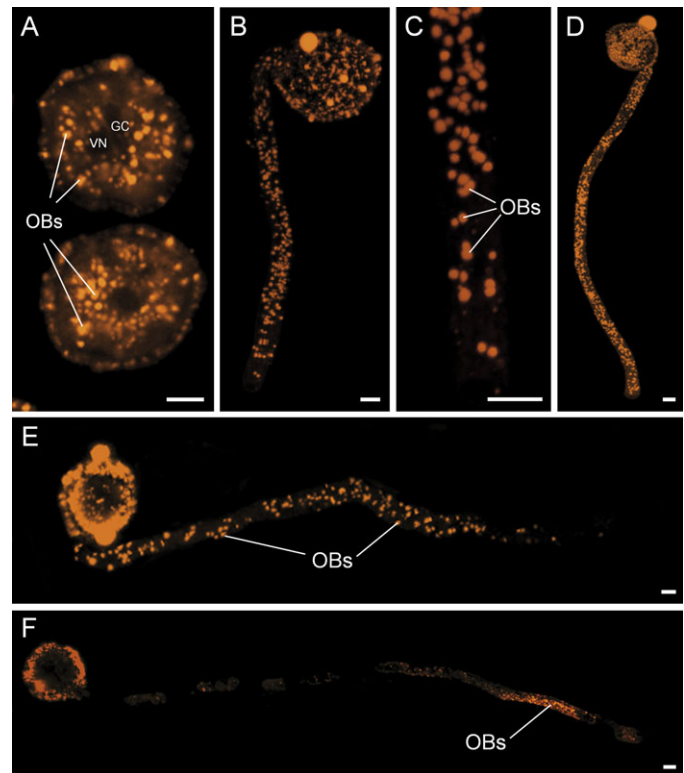


Fig. 3. Nile red staining of OBs from olive pollen. (A) Numerous OBs fill up the cytoplasm of the vegetative cell of the hydrated pollen. The pollen wall is also stained, revealing its lipidic nature. (B–F) OBs are also visible in the pollen tube cytoplasm after 1 (B, C), 3 (D), 6 (E), and 12 h (F) of *in vitro* germination. At higher magnification (C), round-shaped OBs of different sizes, which frequently coalesced, were observed. Images represent Z-stack projections of optical sections obtained using a confocal laser scanning microscope. Bars=10 μm .

cytoplasm of the vegetative cell and was visualized as a yellowish fluorescence (Fig. 6D). Additionally, a specific accumulation of OBs and caleosins was observed in the vicinity of one of the pollen grain apertures (Fig. 6D). During *in vitro* germination, caleosins were located in the boundaries of the OBs spread along the pollen tube and in the cytoplasm at the subapical region (Fig. 6H). Caleosins also located in the tonoplast of the vacuoles present in the pollen tube cytoplasm (Fig. 6L).

At the ultrastructural level, immunogold labelling of caleosins in sections of mature and germinating pollen grains of olive confirmed the results obtained by confocal microscopy. In mature, non-germinated pollen grains, gold particles were mainly found in the cytoplasm of the vegetative cell, attached on the surface of OBs or in the vicinity of these organelles (Fig. 7A, B). Caleosins were also located in the exine, but not in the intine (Fig. 7A). During *in vitro* germination, gold particles were found in the pollen tube cytoplasm and the plasma membrane (Fig. 7C). In the subapical zone, where numerous organelles and membrane structures were present, gold particles were located attached to the surface of OBs (Fig. 7C). Moreover, some gold

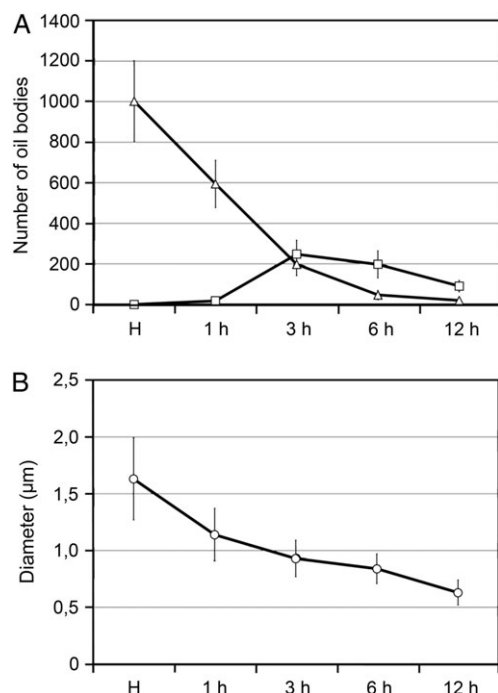


Fig. 4. (A) Changes in the number of OBs within the olive pollen grain (open triangles) and pollen tube (open squares) during *in vitro* germination. (B) Average size of OBs in the olive pollen tube during *in vitro* germination. Z-series images corresponding to the whole volume of five germinated pollen grains from three independent experiments ($n=15$) were captured and used for measurements and statistical analysis. H, hydrated pollen.

particles were found specifically localized in the surrounding ER membranes. In the region of the pollen tube distal from the apex, gold particles were mainly located in the tonoplast of vacuoles (Fig. 7D), which frequently coalesce. A control reaction with omission of the primary Ab did not show any significant gold labeling (Fig. 7E).

Discussion

A number of caleosins have been identified in mature seeds of several plant species such as rice, barley, sesame, sunflower, soybean, rape, and *Arabidopsis* (Frandsen *et al.*, 1996; Chen *et al.*, 1999; Næsted *et al.*, 2000; Jolivet *et al.*, 2004; Liu *et al.*, 2005; Polxeitner *et al.*, 2006). It was only recently that the first caleosin from pollen was identified in lily (Jiang *et al.*, 2008). These authors cloned a cDNA fragment encoding a caleosin of ~27 kDa, whose sequence is distinct from that of those caleosins found in seed OBs. The data presented here constitute, to the best of our knowledge, the first report regarding the identification and cellular localization of a caleosin isoform from pollen in an oleaginous crop plant. The use of an Ab to a heterologous caleosin from *Arabidopsis* (Clo-3 isoform) that is expressed in both vegetative and reproductive tissues (Partridge and Murphy, 2009) allowed identification of a putative caleosin with a molecular mass of ~30 kDa, tightly associated with

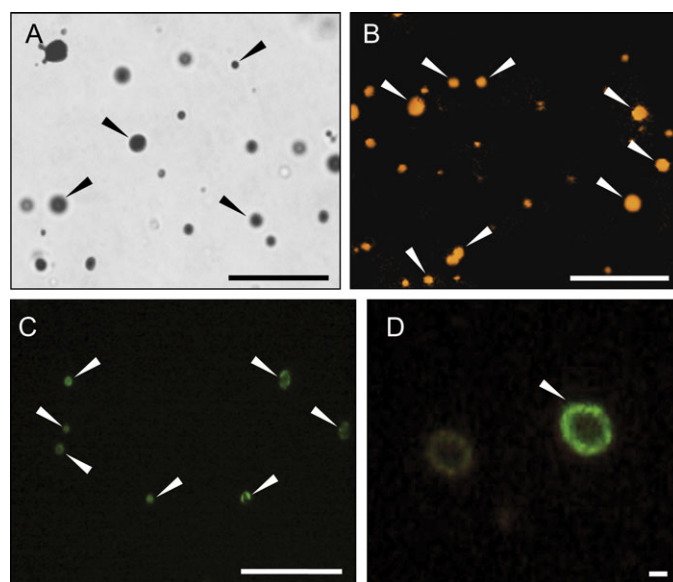


Fig. 5. Purification of OBs from olive pollen and localization of caleosins. (A, B) Purified OBs stained with Sudan Black B and Nile red, respectively. (C, D) Localization of caleosins on isolated OBs using the anti-Clo3 Ab, at low (C) and high (D) magnification. Bars=2 μm.

OBs and ER membranes. However, the possibility of the presence of additional caleosin isoforms in the olive pollen cannot be ruled out. Indeed, two caleosin isoforms, differing in their molecular masses and subcellular localization, co-exist in developing seeds of *Brassica napus* (Hernández-Pinzón *et al.*, 2001).

Expression and localization studies have provided valuable clues about the putative function of caleosins in seeds (Chen *et al.*, 1999; Næsted *et al.*, 2000). The detection of caleosins on purified OBs from olive pollen is in good agreement with the idea that this is an integral membrane protein. Consequently, the olive pollen 30 kDa caleosin was found tightly associated with purified OBs even after vigorous and repeated washes. Interestingly, although the 30 kDa polypeptide was detected during the whole germination process, levels decreased coincidentally with the reduction in the number of OBs present in the cytoplasm of the pollen tube. This pattern was also observed in light-grown cotyledons of *B. napus*, in which levels of the 25 kDa caleosin declined as OBs underwent mobilization (Hernández-Pinzón *et al.*, 2001).

The data presented here also support the hypothesis that OBs move from the pollen grain towards the growing pollen tube as soon as the pollen grain begins to germinate (Rodríguez-García *et al.*, 2003). OB mobilization in the olive pollen started after hydration, and progressed during the subsequent steps of pollen tube elongation. After 12 h of *in vitro* germination, the population of OBs was almost completely metabolized. The function of OBs is still a matter of discussion. It has been proposed that OB-stored TAGs are converted into membrane lipids (Dorne *et al.*, 1988). The localization of caleosins in both the intracellular membranes

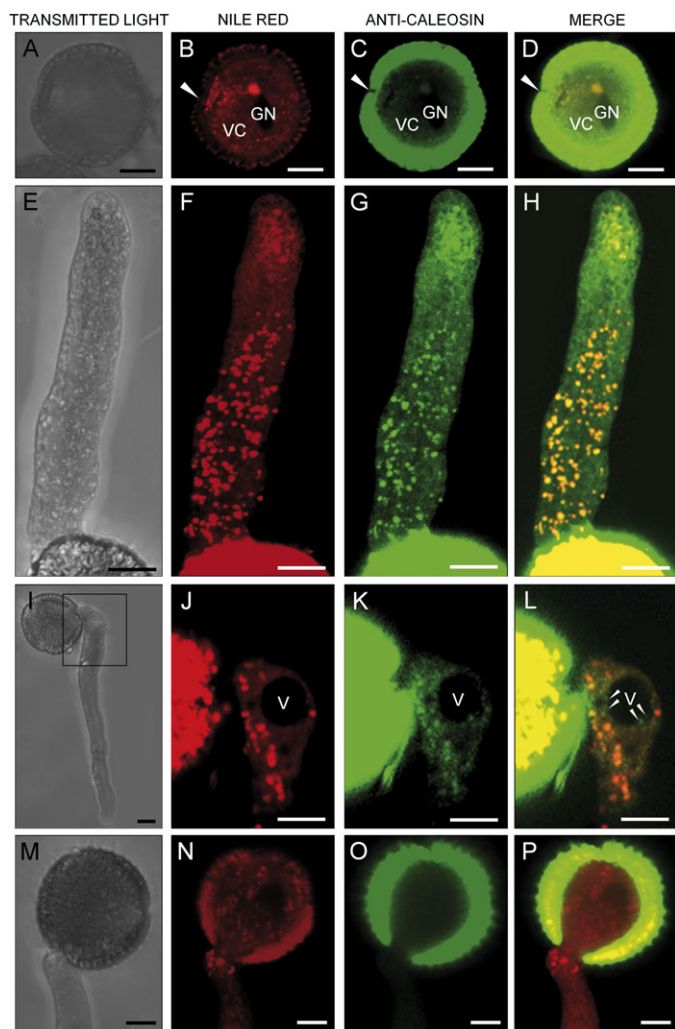


Fig. 6. Staining of OBs and localization of caleosins in olive pollen tubes. (A–D) Optical sections of hydrated pollen grains. The signal corresponding to caleosins and OBs co-localized in the cytoplasm of the vegetative cell and was visualized as a yellowish fluorescence. OBs and caleosins accumulated in the vicinity of one of the pollen grain apertures. (E–H) Optical sections of olive pollen tubes after 2 h of *in vitro* germination. Numerous OBs were found in the pollen tube cytoplasm. Caleosins co-localized with OBs. (I–L) Optical sections of olive pollen tubes after 3 h of *in vitro* germination. Caleosins appeared located at the boundary of the vacuoles and did not co-localize with lipids (arrows). (M–P) Control reaction by omitting the primary anti-Clo3 Ab. GN, generative nucleus; V, vacuole; VC, vegetative cell. Bars=10 μ m.

(e.g. tonoplast) and the plasmalemma is consistent with this idea. Moreover, olive pollen OBs are frequently surrounded by ER membranes (Alché *et al.*, 1999a), which persist during pollen germination (M'rani-Alaoui, 2000). This ER network might facilitate the direct mobilization of the OB lipids into membrane components (Piffanelli *et al.*, 1998).

The function of caleosins in the surface of OBs remains to be studied. Caleosins possess a single EF-hand domain that binds a single Ca^{2+} cation with low affinity. It is therefore possible that a caleosin forms either *cis* or *trans* dimeric

associations with another caleosin present in the same or a distinct membrane or OB, leading to fusion events (Næsted *et al.*, 2000). Accordingly, *AtCLO1* caleosin participates in OB–vacuole interactions that affect breakdown of OBs and turnover of the vacuole membrane as storage vacuoles are remodelled during seed germination in *Arabidopsis* (Poxleitner *et al.*, 2006). In the pollen tube of lily, the absence of glyoxysomes and the presence of OBs inside the vacuoles suggest that degradation of OBs might be carried out by vacuolar digestion (Jiang *et al.*, 2007). Caleosins might therefore mediate OB–vacuole membrane fusion in the pollen tube. Calcium might also directly regulate caleosin activity. In a recent work, Hanano *et al.* (2006) showed that caleosins from *Arabidopsis* and rice seeds possess peroxxygenase activity and that calcium is crucial for this activity. Whether pollen caleosins do possess peroxxygenase activity or not is currently under investigation in our laboratory.

Vacuole formation is a common feature in growing pollen tubes (Noguchi, 1990; Mascarenhas, 1993; Derksen *et al.*, 2002). Interestingly, the mechanism of vacuole biogenesis appears to be different from that in other plant tissues (Hicks *et al.*, 2004). According to the model of Noguchi (1990), OBs enclosed by ER membranes become attached to electron-dense thin vesicles (EDTVs) in the pollen cytoplasm, undergoing lipolysis to disappear completely. In parallel with the consumption of OBs, the formation of protuberances in the expanding EDTVs and the pinching off of these protuberances leads to the formation of developing small vacuoles that fuse to each other and increase in size as they move towards the distal part of the pollen tube. In the olive, the presence of caleosins in the tonoplast of the vacuoles within the pollen tube supports the idea that OBs might be converted into vesicles and vacuoles. Caleosins docked in the tonoplast might mediate vacuole–vacuole fusion events, participating in the reorganization of membrane compartments in the growing pollen tube. Similarly, this mechanism might be mediated by Ca^{2+} ions as described above.

Apart from lipidic material (Pitfanelli *et al.*, 1998), the pollen coat is a site for functional proteins involved in cell wall loosening, pollen hydration, and pollen–stigma communication (Murphy and Ross, 1998; Mayfield *et al.*, 2001; Murphy, 2006). In the olive, the tapetum is involved in the synthesis and accumulation of several proteins that are present in the pollen coat, including some allergens such as Ole e 1 and profilins (Alché *et al.*, 1999b; Morales *et al.*, 2008). Moreover, OB-associated oleosin genes are expressed in the tapetal tissues of this species during anther development (Alché *et al.*, 1999a). In the present work, caleosins were found for the first time in the exine. This finding suggests that caleosins from olive pollen may have both a sporophytic and a gametophytic origin. The analysis of the expression pattern of this caleosin during olive anther development showed that caleosins are also expressed in the tapetum (unpublished results), thus supporting this hypothesis. It has been proposed that these pollen coat-associated caleosins might have a role in pollen–stigma communication

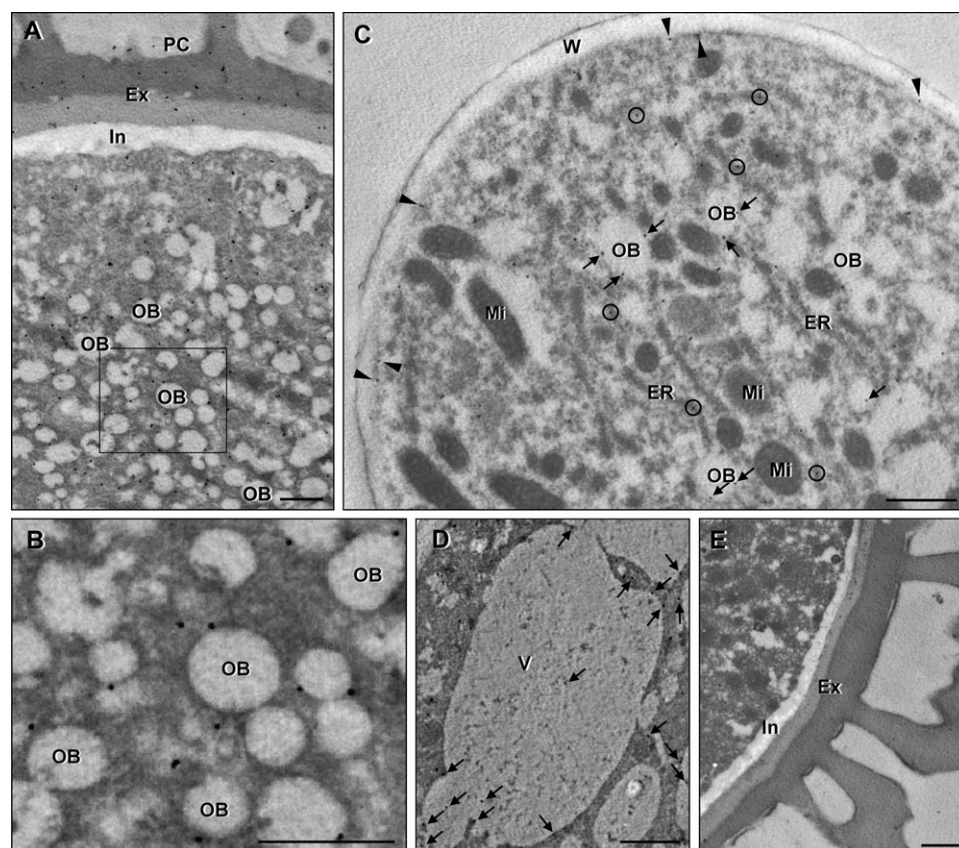


Fig. 7. Ultrastructural localization of caleosins in hydrated and germinated olive pollen. (A) In the hydrated pollen, gold particles were located in the cytoplasm of the pollen grain and in the exine. (B) Higher magnification of boxed area in A, showing gold particles associated with the boundaries of OBs. (C) Section of the subapical region of a pollen tube. Gold labelling was specifically found at the OB boundaries (arrows), the ER (open circles), and the plasma membrane (arrowheads). (D) Section corresponding to the distal region of the pollen tube after 3 h of *in vitro* germination. Large vacuoles (V) filled up the cytoplasm of the pollen tube. Gold particles localized in the tonoplast (arrows). (E) Control reaction by omitting the anti-Clo3 Ab showing the absence of gold labelling. ER, endoplasmic reticulum; Ex, exine; In, intine; Mi, mitochondria; OB, oil body; PC, pollen coat; V, vacuole; W, pollen tube wall. Bars=1 μ m.

during pollination (Murphy, 2006), although this hypothesis needs to be tested in further investigations.

Supplementary data

Supplementary data are available at *JXB* online.

Video S1. Time-lapse-generated movie showing the dynamics of OB motility during early olive pollen *in vitro* germination. Pollen grains were germinated in an appropriate medium for 3 h and OBs were stained with Nile red. Both the transmitted light channel and the resulting fluorescence image were recorded. AVI file.

Video S2. Effect of vacuoles on OB traffic throughout the olive pollen tube cytoplasm during *in vitro* germination. Pollen grains were germinated in an appropriate medium for 3 h and OBs were stained with Nile red. In order to generate the resulting time-lapse movie, both the transmitted light channel and the fluorescence images were recorded. AVI file.

Figure S1. Immunolocalization of caleosins in olive mature pollen sections. (A) Localization of caleosins (green fluorescence) in the cytoplasm of the pollen grain and the exine wall (Ex). The intine layer (arrowheads) and the

apertures (asterisk) were devoid of labelling. (B) Control reaction performed by omitting the primary Ab showing only the exine autofluorescence (in red). Samples were observed with an Axioplan epifluorescence microscope (Carl Zeiss). Digital images were recorded with a ProgRes C3 camera (Jenoptik Laser) using the ProgRes CapturePro software (Jenoptik Laser). Bars=10 μ m.

Acknowledgements

We are grateful to Professor D Murphy (University of Glamorgan, Faculty of Health, Sport and Science, Division of Biology, Pontypridd, UK) for kindly providing us with the anti-Clo3 Ab to caleosin, and to Ms C Martínez-Sierra for expert technical assistance. We would like also to thank IFAPA “Venta del Liano” (Jaen, Spain) for providing olive material. This work was supported by the Junta de Andalucía (project P06-AGR-01791) and the Spanish Ministry of Science and Innovation (projects AGL2008-00517/AGR and BFU2008-00629/BFI). KZ and AZ thank the Junta de Andalucía for providing grant funding. KZ also thanks the CSIC for providing JAE-DOC grant funding.

References

- Alché JD, Castro AJ, Olmedilla A, Fernández MC, Rodríguez R, Villalba M, Rodríguez-García MI.** 1999b. The major olive pollen allergen (Ole e 1) shows both gametophytic and sporophytic expression during anther development, and its synthesis and storage takes place in the RER. *Journal of Cell Science* **112**, 2501–2509.
- Alché JD, Castro AJ, Rodríguez-García MI.** 1999a. Expression of oleosin genes in the olive (*Olea europaea* L.) anther. In: Clément C, Pacini E, Audran JC, eds. *Anther and pollen: from biology to biotechnology*. Berlin: Springer-Verlag, 91–99.
- Alché JD, Jiménez-López JC, Wang W, Castro AJ, Rodríguez-García MI.** 2006. Biochemical characterization and cellular localization of 11S type storage proteins in olive (*Olea europaea* L.) seeds. *Journal of Agricultural and Food Chemistry* **54**, 5562–5570.
- Chen JCF, Tsai CCY, Tzen JTC.** 1999. Cloning and secondary structure analysis of caleosin, a unique calcium-binding protein in oil bodies of plant seeds. *Plant and Cell Physiology* **40**, 1079–1086.
- Derksen J, Knuiman B, Hoedemaekers K, Guyon A, Bonhomme S, Pierson ES.** 2002. Growth and cellular organization of *Arabidopsis* pollen tubes *in vitro*. *Sexual Plant Reproduction* **15**, 133–139.
- Dorne AJ, Kappler R, Kristen U, Heinz E.** 1988. Lipid metabolism during germination of tobacco pollen. *Phytochemistry* **27**, 2027–2031.
- Frandsen G, Müller-Uri F, Nielsen M, Mundy J, Skriver K.** 1996. Novel plant Ca^{2+} -binding protein expressed in response to abscisic acid and osmotic stress. *Journal of Biological Chemistry* **271**, 343–348.
- Frandsen GI, Mundy J, Tzen JTC.** 2001. Oil bodies and their associated proteins, oleosin and caleosin. *Plant Physiology* **112**, 301–307.
- Hanano A, Burcklen M, Flenet M, Ivancich A, Louwagie M, Garin J, Blée E.** 2006. Plant seed peroxygenase is an original heme-oxygenase with an EF-hand calcium binding motif. *Journal of Biological Chemistry* **281**, 33140–33151.
- Hernández-Pinzón I, Patel K, Murphy DJ.** 2001. The *Brassica napus* calcium-binding protein, caleosin, has distinct endoplasmic reticulum- and lipid body-associated isoforms. *Plant Physiology and Biochemistry* **39**, 615–622.
- Hicks GR, Rojo E, Hong S, Carter DG, Raikhel NV.** 2004. Germinating pollen has tubular vacuoles, displays highly dynamic vacuole biogenesis and requires *VACUOLESS1* for proper function. *Plant Physiology* **134**, 1227–1239.
- Huang AHC.** 1996. Oleosin and oil bodies in seeds and other organs. *Plant Physiology* **110**, 1055–1061.
- Jiang PL, Jauh GY, Wang CS, Tzen JTC.** 2008. A unique caleosin in oil bodies of lily pollen. *Plant and Cell Physiology* **49**, 1390–1395.
- Jiang PL, Wang CS, Hsu CM, Jauh GY, Tzen JTC.** 2007. Stable oil bodies sheltered by a unique oleosin in lily pollen. *Plant and Cell Physiology* **48**, 812–821.
- Jolivet P, Roux E, D'Andrea S, Davanture M, Negroni L, Zivy M, Chardot T.** 2004. Protein composition of oil bodies in *Arabidopsis thaliana* ecotype WS. *Plant Physiology and Biochemistry* **42**, 501–509.
- Laemmli UK.** 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 680–685.
- Lin LJ, Tai SSK, Peng CC, Tzen JTC.** 2002. Steroleosin, a sterol-binding dehydrogenase in seed oil bodies. *Plant Physiology* **128**, 1200–1211.
- Lin LJ, Tzen JTC.** 2004. Two distinct steroleosins are present in seed oil bodies. *Plant Physiology and Biochemistry* **42**, 601–608.
- Liu H, Hedley P, Cardle L, Wright KM, Hein I, Marshall D, Waugh R.** 2005. Characterization and functional analysis of two barley caleosins expressed during barley caryopsis development. *Planta* **221**, 513–522.
- Mascarenhas JP.** 1993. Molecular mechanisms of pollen tube growth and differentiation. *The Plant Cell* **5**, 1303–1314.
- Mayfield JA, Fiebig A, Johnstone SE, Preuss D.** 2001. Gene families from the *Arabidopsis thaliana* pollen coat proteome. *Science* **292**, 2482–2485.
- Morales S, Jiménez-López JC, Castro AJ, Rodríguez-García MI, Alché JD.** 2008. Olive pollen profilin (Ole e 2 allergen) co-localizes with highly active areas of the actin cytoskeleton and is released to the culture medium during *in vitro* pollen germination. *Journal of Microscopy-Oxford* **231**, 332–341.
- M'rani-Alaoui M.** 2000. Estudio a nivel celular de la germinación del polen, emisión y elongación del tubo polínico en el olivo (*Olea europaea* L.). PhD thesis, University of Granada, Spain.
- Murphy DJ.** 2001. Biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Progress in Lipid Research* **40**, 325–438.
- Murphy DJ.** 2006. The extracellular pollen coat in members of the *Brassicaceae*: composition, biosynthesis, and functions in pollination. *Protoplasma* **228**, 31–39.
- Murphy DJ, Ross JH.** 1998. Biosynthesis, targeting and processing of oleosin-like proteins, which are major pollen coat components in *Brassica napus*. *The Plant Journal* **13**, 1–16.
- Murphy DJ, Vance J.** 1999. Mechanisms of lipid body formation. *Trends in Biochemical Science* **24**, 109–115.
- Næsted H, Frandsen GI, Jauh GY, Hernandez-Pinzon I, Nielsen HB, Murphy DJ, Rogers JC, Mundy J.** 2000. Caleosins: Ca^{2+} -binding proteins associated with lipid bodies. *Plant Molecular Biology* **44**, 463–476.
- Noguchi T.** 1990. Consumption of lipid granules and formation of vacuoles in the pollen tube of *Tradescantia reflexa*. *Protoplasma* **156**, 19–28.
- Pacini E, Juniper BE.** 1979. The ultrastructure of pollen-grain development in the olive (*Olea europaea* L.). 1. Proteins in the pore. *New Phytologist* **83**, 157–164.
- Partridge M, Murphy D.** 2009. Roles of a membrane-bound caleosin and putative peroxygenase in biotic and abiotic stress responses in *Arabidopsis*. *Plant Physiology and Biochemistry* **47**, 796–806.
- Piffanelli P, Ross JHE, Murphy DJ.** 1998. Biogenesis and function of the lipidic structures of pollen grains. *Sexual Plant Reproduction* **11**, 65–80.
- Poxleitner M, Rogers SW, Samuels AL, Browse J, Rogers JC.** 2006. A role for caleosin in degradation of oil-body storage lipids during seed germination. *The Plant Journal* **47**, 917–933.

Rodríguez-García MI, M'rani-Alaoui M, Fernández MC. 2003. Behavior of storage lipids during development and germination of olive (*Olea europaea* L.) pollen. *Protoplasma* **221**, 237–244.

Ross JHE, Sánchez J, Millán F, Murphy DJ. 1993. Differential presence of oleosins in oleogenic seed and mesocarp tissues in olive

(*Olea europaea*) and avocado (*Persea americana*). *Plant Science* **93**, 203–210.

Tzen JTC, Cao YZ, Laurent P, Ratnayake C, Huang AHC. 1993. Lipids, proteins, and structure of seed oil bodies from diverse species. *Plant Physiology* **101**, 267–276.