

Nanovesicles Are Secreted during Pollen Germination and Pollen Tube Growth: A Possible Role in Fertilization

Dear Editor,

During recent decades, a novel mechanism of secretion has been identified in a wide range of mammalian cells. It involves the release of bioactive membrane nanovesicles (30–100 nm), termed exosomes, upon the fusion of multivesicular bodies with the plasma membrane (Théry et al., 2009). Exosomes are implicated in diverse functions, such as scavenging of archaic proteins, intercellular messengers delivering cell-specific signals, and vehicles for transmissible pathogens. Exosomes have also been described in other organisms such as bacteria, *Drosophila*, and fungi. Concerning exosome-like vesicles in plants, only one study has demonstrated their existence in sunflower apoplastic fluid (Regente et al., 2009), although several pieces of evidence have suggested that they could be involved in cell-wall-associated defense response upon pathogen attack (e.g. Meyer et al., 2009). However, no information is available on exosome-like vesicle release during pollen germination and pollen tube growth, despite the importance of the vesicle trafficking network in such an event (see Moscatelli and Idilli, 2009).

In this study, we show that olive (*Olea europaea*) pollen grains release nanovesicles during in vitro pollen germination and pollen tube growth. We proposed the term ‘pollensomes’ to designate these nanovesicles specifically isolated from germination medium using a protocol previously described for mammalian exosomes (see Methods in Supplementary Data). Electron microscopy analysis revealed a heterogeneous population of round-shaped nanovesicles, ranging from 28 to 60 nm in diameter (Figure 1A). Pollensomes floated at densities of between 1.24 and 1.29 g ml⁻¹ on sucrose gradient, confirming their vesicular nature (Figure 1B). Both sizes and shape agree with those previously reported for exosomes (Théry et al., 2009). Their higher density, compared to mammalian exosomes (1.13–1.19 g ml⁻¹), was consistent with their enrichment in polysaccharides as revealed by Fourier transform infrared (FTIR) analysis: the average FTIR spectrum showed a prominent absorption band between 900 and 1200 cm⁻¹, which is attributed to polysaccharides (Figure 1C). Other absorption bands were detected at: (1) 2800–3000 cm⁻¹, corresponding to the lipid region; (2) 1600–1700 cm⁻¹ and 1525–1575 cm⁻¹, corresponding to amide I and amide II, respectively, which are mainly attributed to the peptide bonds of

proteins, and small contributions of other amide bonds, such as those present in the lipid sphingomyelin; and (3) 1740 cm⁻¹, assigned to saturated esters, is representative of esterified pectins, while the carboxylic acid groups peaking at 1600 and 1414 cm⁻¹ were representative of de-esterified pectins. The presence of pectic material in pollensomes was confirmed by dot blot assay of fractions separated by centrifugation on sucrose gradient, using the monoclonal antibody JIM7 against esterified pectin (Figure 1D). Pectins constitute the main component of the pollen tube wall, particularly at the tip. They are synthesized in the Golgi apparatus and secreted in vesicles as methylesterified polymers, which become de-esterified by the enzyme pectin methylesterase (PME). Pectin network, together to other molecules like arabinogalactan proteins (AGPs), regulate the extensibility and rigidity of the cell wall in growing pollen tubes. Changes in the mechanical and chemical properties of the pollen tube wall have been proposed to be important for its growth process and its interaction with the female floral tissues (reviewed by Palin and Geitmann, 2012). Penetration of pollen tube through the female floral tissues is essential for delivery of the sperm cells to the ovule, so double fertilization can occur.

To gain insight into the proteome of olive pollensomes, proteins were extracted from purified nanovesicles, identified using bottom-up mass spectrometric analysis and categorized in seven different groups based on predicted protein function (see Table 1 and Methods in the Supplementary Data). Pollensomes contain a large number of proteins, many of them displaying well-known roles in *metabolism and signaling* (e.g. fructokinase, glyceraldehyde 3-phosphate dehydrogenase-GADPH- and phosphoglycerate mutase), *protein synthesis and processing* (e.g. peptidyl-prolyl-isomerase), *cell wall expansion* (e.g. PME), *cytoskeleton* (e.g. actin), and *membrane transport* (e.g. H⁺-ATPase 6). All these biological functions may contribute to a coordinated and controlled process responsible for actin cytoskeleton remodeling, cell

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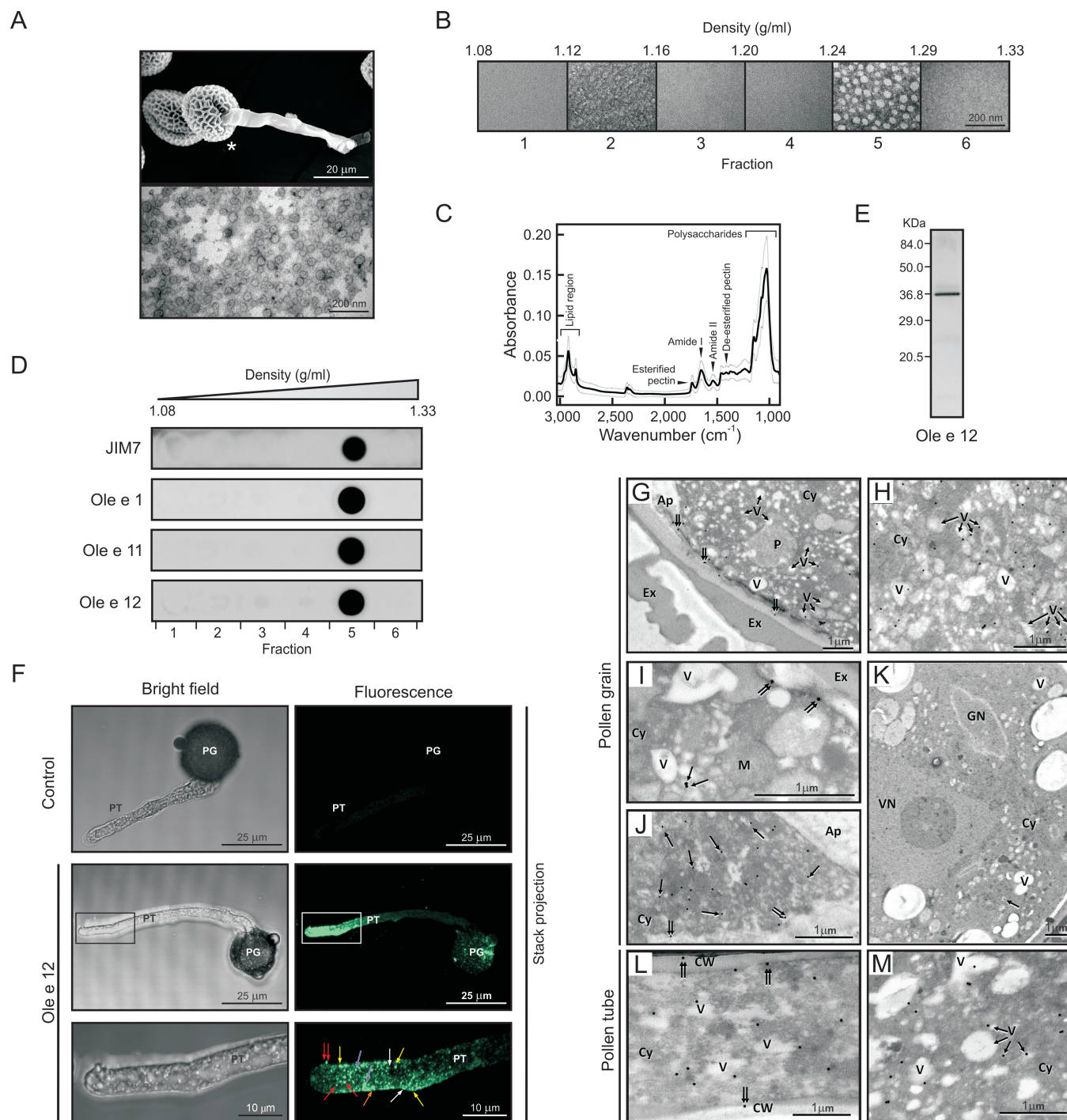


Figure 1. Nanovesicles, Termed Pollensomes, Are Released during In Vitro Pollen Germination and Pollen Tube Growth. Pollensomes were collected at 16 h by filtration and differential centrifugation from the germination media of in vitro-growing pollen tubes. (A) Scanning electron micrograph of a representative olive pollen grain (upper panel). Note the emergence of the pollen tube (asterisk). TEM micrograph of pollensomes released from in vitro-germinated pollen tubes (bottom panel). (B) Pollensomes were subjected to a sucrose gradient and collected fractions were analyzed by TEM. (C) Average FTIR spectrum obtained from olive pollensomes, showing the contribution of lipids, proteins, pectins, and other polysaccharides. Dashed lines represent standard deviations in six independent experiments. (D) Dot blot analysis of fractions separated by centrifugation on sucrose gradient, using the monoclonal antibody JIM7 against esterified pectins, and specific polyclonal antibodies to Ole e 1, Ole e 11, and Ole e 12 allergens. Densities for each fraction are indicated. (E) Western blot analysis of pollensomes expressing Ole e 12, showing the specificity of the rabbit polyclonal anti-Ole e 12 antibody generated against the full-length protein. Pre-stained molecular mass markers (low range Bio-Rad), in kDa, are indicated.

wall expansion, calcium and pH gradients, and energy metabolism, crucial for pollen tube growth and latterly a successful fertilization (Moscatelli and Idilli, 2009). In this sense, several studies support the importance of PME for pollen tube growth towards the ovule, possibly by modification of the cell wall, providing mechanical stability to the pollen tube and promoting the recognition and adhesion between pollen tube and female style and transmitting tract (e.g. Jiang et al., 2005; Castro et al., 2013). Proteins associated with *defense/stress response* were also identified in pollensomes (e.g. phenylcoumaran benzylic ether reductase (PCBER) and heat shock protein 70). Some of them, such as actin, heat shock protein 70, GADPH, and S-adenosyl-homocysteine hydrolase, had been already reported in mammalian exosomes (Théry et al., 2009). A number of proteins with *unknown functions* were also identified in pollensomes.

Interestingly, three of the identified proteins had been previously described and characterized as olive pollen allergens: Ole e 1, Ole e 11 (a PME), and Ole e 12 (a PCBER) (reviewed by Villalba et al., 2013). Ole e 1 belongs to the Ole e 1-like family, which is specifically expressed in pollen. Based on the studies performed with the homologous protein from tomato (LAT52), it has been proposed that Ole e 1 is involved in pollen hydration, pollen tube growth, and pollen–stigma interactions (Muschietti et al., 1994). These functions are consistent with the localization of Ole e 1 in the pollen exine, the subapical and apical regions of pollen tube, and the germination medium (Alché et al., 2004). The release of Ole e 1, Ole e 11, and Ole e 12 associated to pollensomes was confirmed by dot blot, using specific antibodies, after sucrose gradient fractionation of nanovesicles (Figure 1D). Thus, the finding that pollensomes contain a set of proteins involved in (1) pollen hydration, (2) pollen tube growth, and, possibly, (3) pollen–stigma interactions supports a role for pollensomes in fertilization.

To investigate the cellular origin of pollensomes, a combination of confocal laser scanning microscopy (CLSM) and immunogold transmission electron microscopy (TEM) was used. To this end, pollen grains were cultured for 8 h and immunolabeled with the Ole e 12-specific polyclonal antibody as a tracker (see Methods in the Supplementary Data). The specificity of the antibody is shown in Figure 1E. CLSM immunolocalization showed that Ole e 12 is found in association with the pollen exine, and preferentially located in the subapical region of the pollen tubes (Figure 1F). Higher magnification of the apical region localized the protein in

small-rounded organelles, predominantly present in the cytoplasm, close to the tube cell wall, and, sometimes, outside of the pollen tube (double red arrow).

Our ultrastructural studies showed that Ole e 12 was found in the boundaries of numerous vesicles, heterogeneous in size (200–600 nm in diameter), over the cytoplasm of the vegetative cell and, frequently, close to the plasma membrane and to the inner layers of the cell wall (Figure 1G–I, double black arrow). In addition, Ole e 12-containing vesicles were abundantly present in the proximity of the aperture region of pollen grain (Figure 1J) and in the cytoplasm of the pollen tube (Figure 1L and 1M). Some gold labeling was also detected in the vicinity of the pollen tube cell wall (Figure 1L, double black arrow). This is consistent with the participation of the labeled structures in exocytosis events. Moreover, such localization suggests an additional role of PCBER proteins in mediating pollen tube growth.

Pollensomes comprise a heterogeneous population of secretory vesicles, as the differences in the site of synthesis of the molecules that make up their cargo show. It is likely that all of them work together to sustain the apical pollen tube growth. Some of the pollensomes could represent Golgi-derived vesicles, based on PME and pectin contents. Moreover, the Ole e 1 content of pollensomes also suggests an endoplasmic reticulum (ER)-derived origin for them, as the ER has been described as the main place for the synthesis and storage of this protein in olive pollen (Alché et al., 1999). Alternative routes for pollensome release to the culture medium cannot be discarded. Consistently with this is the observation that pollensomes contain several proteins that have been described as putative leaderless secretory proteins (LSP) from plant cells, such as GADPH, S-adenosylmethionine synthetase, and UDP-glucose dehydrogenase, which get access to the extracellular space via unconventional proteins secretion (UPS) (reviewed by Ding et al., 2012). Three endomembrane compartments can mediate UPS in plants: vacuole, exocyst-positive organelle (EXPO), and exosomes. From our data, we cannot conclude that a subset of pollensomes, such as those containing Ole e 12, a leaderless protein, corresponds to exosome-like vesicles. In our ultrastructural studies, inner vesicles have not been detected in Ole e 12-positive compartments from germinating olive pollen, even though different preparation methods for TEM have been used. The application of other specialized methods such as high-pressure frozen/freeze-substitution employed to describe EXPOs in *Arabidopsis thaliana* (Wang et al., 2010) could improve

(F) Immunofluorescence localization of Ole e 12 allergen in an olive pollen grain and a pollen tube grown in vitro for 8 h. Negative control (upper panel) and low magnification immunolocalization of Ole e 12 (medium panel). Stack projection of 16 individual sections from high-magnification boxed area (bottom panel). Colored arrows point well-individualized particles in the stack projection. Note a fluorescent particle outside the pollen tube (double red arrow). PG, pollen grain; PT, pollen tube.

(G–M) Ultrastructural localization of Ole e 12 allergen in an olive pollen grain (G–J) and a PT grown in vitro for 8 h (L, M). Ole e 12, represented by gold particles, was specifically found on vesicles and in the cell wall (double arrows). (J) Note the accumulation of gold particles close to the aperture region. (K) Negative control without primary antibody. Ap, aperture; CW, cell wall; Cy, cytoplasm; Ex, exine; GN, generative nucleus; M, mitochondria; P, plastid; V, vesicle; VN, vegetative nucleus.

the level of ultrastructural detail. Finally, recent work by [Castro et al. \(2013\)](#) provides new information on the map of pectins and AGPs in the olive pollen tube cell wall, confirming the new synthesis and secretion of pectins and AGPs mainly during late stages of pollen germination. Pectins are present in the apertures, sometimes like a collar-like structure surrounding the germinative aperture, and along the pollen tube, particularly at the tip, with regular ring-shaped fluctuations in pectin deposition, and like spots irregularly distributed along the outer surface of the pollen tube wall. Some of these morphological features are compatible with the presence of vesicles. At the ultrastructural level, pectins localized in both the inner and the outer layers of the pollen tube cell wall, suggesting their migration throughout the pollen tube cell wall (eventually to reach the extracellular medium), may occur. However, it is still to be demonstrated that pollensomes may act like vehicles for such transport, perhaps through a not fully organized cell wall, like that of the pollen tube tip. Pollenosome-driven release of molecules to the extracellular space during pollen tube growth does not seem to be the only existing mechanism for this purpose in pollen. On the view of the numerous questions raised, much work will be required to elucidate the origin and the biological significance of these nanovesicles for pollen.

The authors are aware that our findings deserve further characterization, but we provide a novel insight into the dynamics of protein secretion during the processes of pollen germination and pollen tube growth, which are critical for successful fertilization in flowering plants. Moreover, the presence of allergens in pollensomes strongly suggests their active role in allergic responses in humans, which adds to our work.

SUPPLEMENTARY DATA

Supplementary data are available at *Molecular Plant Online*.

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Running title: Nanovesicles secreted in germinating pollen

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SUPPLEMENTARY DATA

METHODS

Pollen material

Mature pollen grains were collected from olive (*Olea europaea*) trees in Granada (Spain) during seasons from 2009 to 2011. Pollen was dried overnight and stored at -20°C until used.

Antibodies and reagents

Polyclonal sera against Ole e 1, Ole e 11 and Ole e 12 were generated by the laboratory of Dr. F. Vivanco (Fundación Jiménez Díaz, Madrid, Spain). The anti-pectin monoclonal antibody JIM7 was kindly provided by Dr. K. Roberts (John Innes Center, Norwich, UK). Horseradish peroxidase-conjugated goat anti-rabbit IgG (BioRad, <http://www.bio-rad.com>), goat anti-mouse IgG (Pierce Chemical Co, <http://www.piercenet.com>) and rat anti-mouse IgG (Bio-Yeda, <http://www.yedarnd.com>) were used as secondary antibodies. Goat anti-rabbit IgG-Alexa 488 and gold (30 nm)-coupled secondary antibodies were from Molecular Probes (www.invitrogen.com) and BBInternational (<http://www.buybbi.com>), respectively.

In vitro pollen germination and isolation of exosomes-like vesicles

Olive pollen (0.1 g) was hydrated in a humid chamber at room temperature for 30 min before transferring to Petri dishes (15 cm in diameter) containing 20 ml of germination medium: 10% sucrose, 0.03% Ca(NO₃)₂, 0.01% KNO₃, 0.02%

MgSO₄ and 0.03% H₃BO₃. Pollen was germinated at 30°C in dark for 16 h (Alché et al., 2004).

Germination medium was collected and cleared of pollen debris by centrifugation. Exosome-like vesicles were isolated from cleared medium by two successive filtrations on 0.22 µm filters, followed by ultracentrifugation at 100,000g as previously described (Prado et al., 2008). Protein concentration in exosome-like vesicles preparations was measured by the micro-bicinchoninic acid assay (Pierce).

In some experiments, exosome-like vesicles were layered on top of a sucrose gradient (0.58 M-2.0 M sucrose in 20 mM Tris-HCl, pH 7.2) and centrifuged at 45,000 rpm (SW65 rotor, Beckman Instruments, <https://www.beckmancoulter.com>) for 20 h (Prado et al., 2008). Fractions (0.75 ml) were collected from the top of the tube, washed and analyzed by TEM and dot blot.

Western blot and Dot blot analysis

Proteins from pollen derived-nanovesicles were resolved by 17% SDS-PAGE and analyzed by Western blot or dot blot as described (Batanero et al., 1996).

Scanning electron microscopy

Pollen grains were dusted on aluminum stubs and covered with 200 µl of germination medium. After evaporation of the fluid at room temperature, pollen preparations were sputter-coated with gold. Images were obtained at 80 kV with a JEOL 6400 scanning electron microscope (JEOL, <http://www.jeol.com>) and

images collected and processed with the software INCA (<http://www.oxford-instruments.com>).

Transmission electron microscopy: ultrastructure and immunolocalization

For ultrastructural observations, nanovesicles were fixed in 2% paraformaldehyde and loaded on copper-formvar/carbon-coated 200-mesh electron microscopy grids. Samples were contrasted with 1% uranyl acetate.

In vitro germinated pollen samples were processed for TEM and immunogold labeling as described (Alché et al., 1999). Ultrathin sections were transferred on to formvar-coated nickel 300-mesh electron microscopy grids. All incubations were performed at room temperature. Samples were blocked with PBS-5% BSA for 15 min, and then incubated for 2.5 h with a polyclonal antibody against Ole e 12 (1:50). After washing in PBS, samples were incubated with the gold coupled-secondary antibody goat anti-rabbit IgG (1:100) for 2 h. Finally, samples were washed in PBS, rinsed in double-distilled water, and contrasted with 5% uranyl acetate for 5 min, prior to observation. Samples were processed in duplicate and negative controls were performed by omitting primary antibodies. All TEM observations were carried out at 80 kV with a JEOL1011 transmission electron microscope (JEOL) and images collected and processed with the software Soft Imaging Viewer (<http://www.soft-imaging.net>).

Confocal laser scanning microscopy and immunolocalization

In vitro germinated pollen samples were processed and immunolabeled for CLSM according to Zienkiewicz et al. (2010). Germinated pollen grains were separated from the culture medium by centrifugation at 5,000g for 10 min, and

fixed overnight with 4% paraformaldehyde/0.2% glutaraldehyde in 0.2 M Na-cacodylate buffer (pH 7.2) at 4°C. After three washes, samples were digested with 1% cellulase R10 (Serva, <http://creschem.com>) and pectinase (27 U/ml, Sigma, <http://www.sigmaaldrich.com>) in 10 mM citrate buffer (pH 4.8) for 30 min at 37°C, then washed and blocked with PBS-1% BSA for 10 min at 4°C. Samples were washed three times in PBS and incubated overnight with the anti-Ole e 12 primary antibody (1:100 in PBS-1% BSA) at 4°C. Samples were then washed and incubated with the secondary antibody goat anti-rabbit IgG-Alexa 488 (1:200 in PBS-1% BSA) for 2 h, in the dark, at 4°C. Finally, samples were washed and mounted with citifluor (Sigma). Primary antibody was omitted in negative controls. Fluorescence was visualized with a Nikon C1 confocal microscope (Nikon, <http://www.nikoninstruments.com>) using a He/Ne (543 nm) laser. Images were obtained and processed with the software EZ-C1 Gold version 2.10 build 240 (Nikon).

Fourier transform infrared analysis

In a thin film on the Ge plate, 20 µl of a suspension of nanovesicles (0.5 mg/ml in water) were dried under a nitrogen flow. FTIR spectra were recorded on an IFS 66v/S FTIR spectrophotometer (Bruker, <http://www.bruker.com>) equipped with a DTGS detector, with a total of 256 scans per sample at a resolution of 2 cm⁻¹ as described before (Alegre-Cebollada et al., 2008). Dry air was used to purge the optic and the sample compartments (15 l/min). Two levels of zero filling of the interferogram were performed before Fourier transform. The resulting spectra were corrected for atmospheric water absorption lines and smoothed by apodization of its Fourier transform with the Fourier transform of a

4 cm⁻¹ Gaussian line shape (Goormaghtigh and Ruyschaert, 1994). Spectra were obtained in triplicate and six independent experiments were performed.

Bottom-up mass spectrometry and *de novo* sequencing

Proteins (15 µg) from pollen-derived nanovesicles were precipitated in the presence of 6% TCA and 2 µl of a saturated solution of sodium deoxycholate, washed with ice-cold 80% acetone, and digested with trypsin (Promega) as described previously (Casado-Vela et al., 2011). Peptides were concentrated, desalted on a Zorbax 300SB-C18 cartridge (Agilent Technologies, <http://www.home.agilent.com>) and separated on a Zorbax 300SB RP-C18 column (Agilent Technologies) using an Agilent 1200 HPLC system. Peptides were detected and fragmented with an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, <http://www.thermofisher.com>) operated at high resolution to enable *de novo* peptide sequencing. Database searches were performed against NCBI nr database including all organisms and taxonomies. The following parameters were used for the searches: enzyme, trypsin; one missed cleavage allowed; carboxyamidomethyl cysteine as fixed modifications; oxidation of methionine as variable modification. Confident peptide identifications were achieved by selecting narrow ion tolerances (5 ppm for precursor ions and 0.005 Dalton for fragment ions) combined with restricting the list of peptide candidates to those with ≤1% false discovery rate. Fragment spectra bearing structural information on peptides were sequenced *de novo* using PEAKS software (www.bioinformaticssolutions.com). Only those peptide sequences with peptide score ≥ 50, quality values ≥ 0.75 and with at least four amino acids with confidence ≥ 99.5% were considered valid candidates. The

1 resulting peptide sequences were manually confirmed and the cognate proteins
2 identified by homology using Blast (Altschul et al., 1993). The pool of peptide
3 information acquired may be valuable for future pollensome characterization.
4 Therefore, mass spectrometric data were converted to XML format using
5 PRIDE converter v.2.5.3 and uploaded to the Proteomics Identification
6 Database (PRIDE) public data repository (www.ebi.ac.uk/pride/). The name of
7 the files and the accession numbers are: Navovesicles secreted during pollen
8 germination (accession number 18857, including 10427 MS/MS spectra
9 deposited). Information regarding sample preparation, mass spectrometric
10 analysis and software processing was included using Ontology Lookup Service
11 controlled vocabulary, as recommended by the MIAPE guidelines (Taylor et al.,
12 2007).

Table 1. Peptides from olive pollensomes identified after *de novo* sequencing and attributed proteins.

Protein description identity	Peptide(s) <i>de novo</i> sequenced	Peaks score (%)	Blast protein e-value	Homologue peptide sequence found in other species and similarity (%)
<i>Cytoskeleton-related proteins</i>				
Actin-1	AEYDESGPS(I/L)VHR	99.99	0.012	AEYDESGPSIVHR <i>Zea mays</i> (100)
Actin	FME(I/L)PDGQV(I/L)T(I/L)GAER	99.75	0.039	ELPDGQVITIGAER <i>Camellia sinensis</i> (85)
<i>Cell wall expansion</i>				
UDP-glucose dehydrogenase	(I/L)S(I/L)YDPQVTEQ(I/L)QR	99.99	6e ⁻⁴	LSIYDPQVTEQ(I/L)QR <i>Arabidopsis thaliana</i> (86)
Pectin methylesterase precursor (Ole e 11)*	(I/L)YGFQDT(I/L)NC _{cam} DK	99.81	26	YGFQDTL-CD <i>Olea europaea</i> (90)
<i>Defence/stress-related proteins</i>				
Monodehydroascorbate reductase	G(I/L)SDFGVQQDAK	81.16	111	LSDFGVQQGADAK <i>Cucumis sativus</i> (83)
	(I/L)SDFGVQGADAK	80.70	0.053	LSDFGVQQGADAK <i>Cucumis sativus</i> (100)
	WGAF(I/L)ESGTEEPNK	90.07	0.095	WGAFLESGTPEENK <i>Camellia sinensis</i> (86)
Monodehydroascorbate reductase (NADH)-like protein	(I/L)EDDADK(I/L)VEA(I/L)K	99.11	2.4	DDADKLVEAIK <i>Arabidopsis thaliana</i> (90)
5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	FA(I/L)ESFWDGK	99.99	0.23	FALESFWDGK <i>Zea mays</i> (100)
	(I/L)PSTEE(I/L)ADR	99.98	112	PSTEEIADR <i>Zea mays</i> (88)
	(I/L)YDWAVHSFR	77.40	11	DWAVHSFR <i>Zea mays</i> (100)
NBS-LRR type disease resistance protein	QLDS(I/L)VADR	98.25	135	QLDSLVGDR <i>Ipomoea batatas</i> (88)
Phenylcoumaran benzylic ether reductase (Ole e 12)*	VAFNEENDLGTYT(I/L)K	99.69	0.56	FNEEHDIGTYTIK <i>Nicotiana tabacum</i> (76)
Immediate early fungal elicitor protein CMPG1	A(I/L)DADNAAE(I/L)(I/L)AATNK	95.96	14	LSADDAAELLAA <i>Zea mays</i> (83)
<i>Membrane transport</i>				
Arabidopsis H ⁺ -ATPase 6	HHEQEAA(I/L)(I/L)VPGD(I/L)(I/L)S(I/L)K	99.99	0.012	EQEAAILVPGDLISIK <i>Arabidopsis thaliana</i> (80)
Arabidopsis H ⁺ -ATPase 7	(I/L)DQSA(I/L)TGES(I/L)PVTK	99.49	9e ⁻⁴	DQSALTGESLPVTK <i>Arabidopsis thaliana</i> (100)
Putative plasma membrane ATPase	(I/L)PVEQVFEQ(I/L)HHR	99.99	25	PVEEVFEQL <i>Arabidopsis arenosa</i> (88)
Sorbitol transporter	(I/L)APVYSAEVSWWR	95.16	19	APVYSAEVS <i>Malus x domestica</i> (100)
Mannitol transporter	(I/L)TEDVEE(I/L)FGTFFR	99.17	0.23	EEVEEVFGTFFR <i>Artemisia annua</i> (83)
Monosaccharide transporter 2	MPTGVDEEYND(I/L)(I/L)NAS(I/L)ESQK	82.83	1.8	VDEEFNDLVAASEESRK <i>Olea europaea</i> (70)
Monosaccharide transporter	QQNVDEEYSD(I/L)(I/L)EASEAAK	100	0.016	NVDEEFNDLVEASEKAK <i>Populus tremula x Populus tremuloides</i> (76)
Glucose transporter	NVDDVDQEFMD(I/L)(I/L)NAS(I/L)ESQK	99.98	0.73	VDDVSQEFDDLVAASKESQ <i>Arabidopsis thaliana</i> (73)
Sugar transport protein	TSDS(I/L)EDAQ(I/L)R	91.57	1.4	TSDSLEEAQLR <i>Coffea canephora</i> (90)

Putative ammonium transporter	TSGNYYGQWSVAGR	99.51	0.095	TSGSYYGQWSAVGR <i>Camellia sinensis</i> (78)
<i>Metabolism & Signalling</i>				
Adenosylhomocysteinase	EDT(I/L)QEYWW _{ox} NER	99.56	2.4	ETLQEYWWCSER <i>Medicago truncatula</i> (75)
	WVFPETK	99.99	58	WVFPETK <i>Medicago truncatula</i> (100)
Aspartate aminotransferase	(I/L)(I/L)FGADSV _A (I/L)QENR	99.99	1.8	LIFGADSPAIQENR <i>Triticum aestivum</i> (78)
Alpha-1,4-glucan-protein synthase	QSPGFDYE(I/L)YNR	99.94	1	GFDYELYNR <i>Zea mays</i> (100)
Beta-fructofuranosidase	C _{cam} PPSYT(I/L)GTYDTEK	99.99	14	YTLGTYDTKK <i>Arabidopsis thaliana</i> (90)
2,3-biphosphoglycerate-independent phosphoglycerate mutase	(I/L)DQ(I/L)Q(I/L)(I/L)(I/L)K	93.54	9.6	LDQLQLLLK <i>Zea mays</i> (100)
	(I/L)YEGEGFNY(I/L)K	98.43	404	LYEGEGFKYIK <i>Prunus dulcis</i> (81)
	TSGEY(I/L)VHD(I/L)GR	99.93	34	TSGEYLVHN <i>Ricinus communis</i> (88)
	YFVKDEDNS(I/L)HP(I/L)(I/L)A(I/L)YQDK	99.99	0.3	FFVRDEDGASMGPLLALLQDK <i>Nicotiana tabacum</i> (66)
Farnesyl pyrophosphate syntethase	YS(I/L)S(I/L)HR	99.99	341	YSLSLHR <i>Olea europaea</i> (100)
Fructokinase	APGGAPANVALAVAR	96.34	0.004	APGGAPANVAIAVAR <i>Arachis hypogaea</i> (100)
	(I/L)DDASAM _{ox} SLWHDN(I/L)K	99.99	0.016	DDATAMSLWHPNLK <i>Beta vulgaris</i> (85)
	(I/L)P(I/L)WPSADEAR	99.80	0.23	LPLWPSAEEAR <i>Beta vulgaris</i> (90)
	TPDELNLE(I/L)(I/L)R	96.26	4.4	TPDELNLDLIR <i>Beta vulgaris</i> (81)
Fructokinase-1	TA(I/L)AFVT(I/L)R	99.99	17	TALAFVTLR <i>Zea mays</i> (100)
Fructokinase-like protein	SSSHGSVDAFHVK	99.80	7.9	HGSVDAFHV <i>Cicer arietinum</i> (100)
Glyceraldehyde 3-phosphate dehydrogenase	VPTVDVSVVA(I/L)TDR	96.02	0.007	VPTVDVSVVDLTDR <i>Mallotus resinus</i> (92)
Isopentenyl diphosphate isomerase	(I/L)ESEN(I/L)(I/L)HR	87.54	42	ESENLLHR <i>Solanum lycopersicum</i> (100)
Phosphofructokinase V11	FGPQ(I/L)(I/L)SNVQSC _{cam} A(I/L)SAEK	99.97	25	QLISNV-CTDALSAEK <i>Gossypium hirsutum</i> (70)
Pyrophosphate-dependent phosphofructokinase alpha subunit	(I/L)TLFD(I/L)TK	99.99	67	LTLFDLTK <i>Citrus sinenses</i> x <i>Poncirus trifoliata</i> (100)
	NQFVETNVGFDQVM _{ox} K	96.99	0.039	NQFVETNVGFD <i>Citrus sinenses</i> x <i>Poncirus trifoliata</i> (100)
Phosphoglycerate mutase	A(I/L)EYDDFDKFDR	99.99	0.009	ALEYEDFDKFDR <i>Solanum tuberosum</i> (91)
NADP-dependent malic enzyme	FAHHSGSPFDPVEYDGK	98.41	4 e ⁻⁴	FA-SGSPFDPVEYDGK <i>Oryza sativa</i> (88)
	SPAVKYQNPPSAVFTQE(I/L)QEK	97.76	1.8	PPS-VFNQELQEK <i>Phaseolus vulgaris</i> (84)
Putative S-adenosylmethionine synthetase	A(I/L)GVAEP(I/L)SVFVDITYK	94.10	5x10 ⁻⁵	AIGVAEPLSVFVDITYK <i>Capsicum annuum</i> (100)
	(I/L)GGFTSADVGLDADK	98.83	0.053	GFTSADVGLDAD <i>Capsicum annuum</i> (100)
S-adenosyl-L-methionine synthetase	V(I/L)VN(I/L)EQQSPDRSYAK	95.66	1	VLVNIEQQSPD <i>Chrysanthemum coronarium</i> (90)
S-adenosyl-homocysteine hydrolase	EGTLQEYWW _{ox} NER	86.55	2.4	EGETLQEYWWCTER <i>Nicotiana suaveolens</i> (78)
	VWM _{ox} GRTNSG(I/L)(I/L)V(I/L)AEGR	89.86	7.8	TNSGIIVLAAGR <i>Nicotiana benthamiana</i> (83)
	GKTFPDTNSG(I/L)(I/L)V(I/L)AEGR	99.48	0.005	FDPTNSGIIVLAAGR <i>Nicotiana benthamiana</i> (86)
	TEFGPSQPFK	95.71	0.56	TEFGPSQPFK <i>Petunia x hybrida</i> (100)

Serine hydroxymethyltransferase	G(I/L)(I/L)EA(I/L)GSA(I/L)TNK	98.79	4.4	MEALGSALTNK <i>Zea mays</i> (90)
<i>Protein synthesis and processing</i>				
Heat shock protein 70	YTDASVQSD(I/L)K	99.90	11	FTDASVQSDMK <i>Camellia sinensis</i> (81)
Peptidyl-prolyl isomerase	(I/L)VDVC _{cam} NST(I/L)GDFNDAR	99.62	61	LGDFNDAR <i>Zea mays</i> (100)
<i>Unknown proteins</i>				
Unnamed protein product	C _{cam} WQDVFW(I/L)TQGK	100	34	WRDVFWLT <i>Vitis vinifera</i> (87)
Unnamed protein product	M _{ox} KGQTNYAQSDYQRNM _{ox} K	95.33	34	NYAQTSYDQR <i>Vitis vinifera</i> (90)
Unnamed protein product	DFEQ(I/L)AEF(I/L)HR	99.87	0.23	DFEQIAEFLHR <i>Vitis vinifera</i> (90)
Unnamed protein product	DKPSFGVEASE(I/L)YDPVK	84.1	2e ⁻⁶	KPSFGVEASELYDPVK <i>Vitis vinifera</i> (100)
Unnamed protein product	EVSGEAGQQ(I/L)DKAYYK	99.26	7.8	VDGEAGQQLDK <i>Vitis vinifera</i> (90)
Unnamed protein product	FPGDTH(I/L)GADDFDNR	98.85	0.009	GDTHLGDDFDNR <i>Vitis vinifera</i> (92)
Unnamed protein product	(I/L)PTN(I/L)GWR	99.96	15	LPTNLGWR <i>Vitis vinifera</i> (100)
Unnamed protein product	MPYQE(I/L)SE(I/L)AEQAK	99.57	1.3	YRELSEMAEQAK <i>Vitis vinifera</i> (83)
Unnamed protein product	VTSPDVYAVGVWTFP(I/L)K	93.69	0.41	TSVPDVYAVGDVATFPLK <i>Vitis vinifera</i> (83)
Unnamed protein product	TEDGVDN(I/L)FER	98.00	0.053	TEDGVDNLFER <i>Vitis vinifera</i> (100)
Unnamed protein product	VED(I/L)GALH(I/L)(I/L)PK	99.38	2.4	VEDIGALHLIPK <i>Vitis vinifera</i> (83)
Hypothetical protein	DMWSGGPVTGNTDGMNAK	93.81	81	MWSV-VTGYTGGMN <i>Oryza sativa</i> (66)
Hypothetical protein	QGDWFFRYR	100	135	WFFRYR <i>Oryza sativa</i> (100)
Hypothetical protein	GPF(I/L)AAEHNK	100	485	FLAAEHN <i>Vitis vinifera</i> (100)
Hypothetical protein	HVMKSNDV(I/L)NAR	76.82	149	MKSNDILDA <i>Vitis vinifera</i> (77)
Hypothetical protein	QRRYDKETWR	100	83	QRRY-ETWR <i>Vitis vinifera</i> (80)
Hypothetical protein	NMGKAVEVR	99.99	100	NMGKYAVEVR <i>Vitis vinifera</i> (90)
Hypothetical protein	VA(I/L)NTGG(I/L)(I/L)SSKTQMP(I/L)NK	82.90	7.7	VVL-GGLLSSKKQLPTVWEQVLNK <i>Vitis vinifera</i> (56)
Hypothetical protein	(I/L)AAQADAGNTPVNVK	98.71	1.3	AAQADAGNTPNNTK <i>Nicotinia tabacum</i> (85)
Hypothetical protein	VAGG(I/L)VDENEFT	94.93	46	GGLVEENEFT <i>Zea mays</i>
Hypothetical protein OsJ_007152	A(I/L)GEQ(I/L)SNLEAQ(I/L)DTVR	77.26	2.4	ALGEQLSAEEDAAVMAEFENLEAQL <i>Oryza sativa</i> (52)
Hypothetical protein OsJ_012416	AT(I/L)GYDQST(I/L)NT(I/L)SM _{ox} NK	99.92	0.33	LGYDQRTLNTLS <i>Oryza sativa</i> (91)
Hypothetical protein OsI_029502	EGTTTRR(I/L)WR	97.10	34	GTTTLRRIW <i>Oryza sativa</i> (88)
Hypothetical protein OsJ_021719	DEWKSSPPEQVSQR	100	46	DEWKSSQPE <i>Oryza sativa</i> (88)
Hypothetical protein OsJ_032488	(I/L)AMGVQHD(I/L)(I/L)GVEAK	99.32	34	LALSVLHDLLGVAA <i>Oryza sativa</i> (71)
Hypothetical protein OsJ_008044	SPPAVDA(I/L)VKPVNPDAK	80.84	4.3	AVSSDAAVKPVNPDA <i>Oryza sativa</i> (80)
Hypothetical protein OsI_031500	RWADGAVTYVGVGGYGK	84.49	34	YIVGIGGYGK <i>Oryza sativa</i> (80)
Hypothetical protein OsI_032491	SRQHVNE(I/L)E(I/L)QNT(I/L)SK	99.99	10	HVNELE-ENTLS <i>Oryza sativa</i> (83)
Hypothetical protein OsJ_024412	TPPWQDT(I/L)C _{cam} DNK	100	111	WQDTLCD <i>Oryza sativa</i> (100)

Hypothetical protein Osl_018180	VFRNESGFNPM _{ox} HH(I/L)VER	94.63	19	RN-SGFSPKMDHLV <i>Oryza sativa</i> (71)
Hypothetical protein OsJ_007305	WTDVQYEVSEAPTR	76.33	34	DV-YEVSEAQTR <i>Oryza sativa</i> (83)
Hypothetical protein OsJ_011052	VYDRTAYYFNK	98.60	14	VYEDRSEYYFNK <i>Oryza sativa</i> (75)
Hypothetical protein Osl_39293	YQWDQYET(I/L)QKKGPMC _{cam} K	98.37	25	DQYETLQRK <i>Oryza sativa</i> (88)
Hypothetical protein OsJ_025879	VDQ(I/L)SEM(I/L)VPGR	100	26	VDQLTEMLV <i>Oryza sativa</i> (88)
Hypothetical protein OsJ_002739	WV(I/L)QDHEDDV(I/L)K	99.21	11	WVLQDHEPDD <i>Oryza sativa</i> (90)
Hypothetical protein Osl_13	YDYENV DAGQK	98.68	2.4	YDYENV DAG <i>Oryza sativa</i> (100)
Hypothetical protein 27.100104	SQRRC _{cam} VR	100	189	SQRRCVR <i>Brassica oleracea</i> (100)
Unclassified proteins (Miscellaneous)				
Major olive pollen allergen Ole e 1*	AEG(I/L)YSM _{ox} (I/L)VER	99.99	0.053	AEGLYSMLVER <i>Olea europaea</i> (100)
	DQFC _{cam} E(I/L)T(I/L)(I/L)SSSR	99.84	0.55	NEFCEITLLSSSR <i>Olea europaea</i> (76)
Branched silkless1 (putative ERF-transcriptor factor)	F(I/L)APFHHTKR	99.45	149	FLAPFHH <i>Zea mays</i> (100)
CBF-like transcription factor	TNPEYN(I/L)TPSTR	83.20	62	TGPEYNLTP <i>Avena sativa</i> (88)
PHD finger family protein	YFNDGD(I/L)VWT(I/L)VK	100	2.4	NDGDLVWTL <i>Arabidopsis thaliana</i> (100)
Sulfotransferase family protein	VSGT(I/L)WLK	98.20	390	SGTLWLK <i>Arabidopsis thaliana</i> (100)
T2E6.21	MPTK(I/L)GS(I/L)VQRQ(I/L)SSSK	99.49	14	MNTFFKLGSLIQRTASQISSS <i>Arabidopsis thaliana</i> (61)
WERBP-1	RED(I/L)GN(I/L)K	89.89	390	REDLGNL <i>Nicotiana tabacum</i> (100)
RHM1	EEDTPNF(I/L)GSFYSK	99.99	6e ⁻⁴	EEDTPNFIGSFYSK <i>Zea mays</i> (100)
Hypothetical chloroplast RF1	QWWG(I/L)S(I/L)VSYGK	87.70	34	WWGLR-VSYGK <i>Pelargonium x hortorum</i> (81)

*Proteins previously described as allergens.

C_{cam}: carbamylomethylated Cys.

M_{ox}: oxidised Met.

(I/L): Ile and Leu, share the same monoisotopic mass and, thus, cannot be distinguished in *de novo* sequence experiments using mass spectrometry. Accordingly,

(I/L) represents that either Ile or Leu may suit in those specific positions where this symbol is included.

(-): gap included in peptide sequences using Blast algorithm (BLOSUM62 scoring matrix).

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