

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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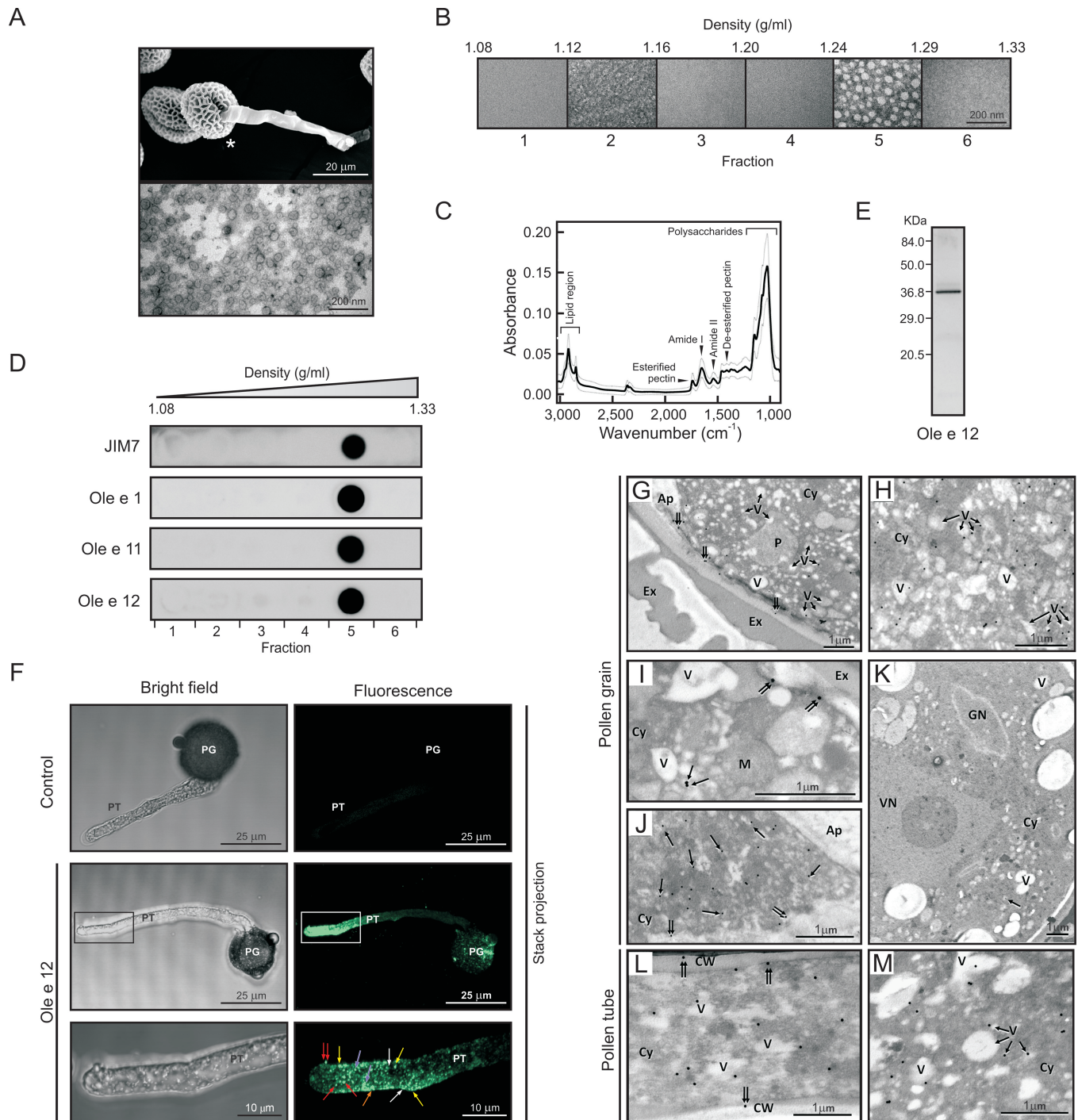


Figure 1. Nanovesicles, Termed Pollensomes, Are Released during In Vitro Pollen Germination and Pollen Tube Growth.

Pollensomes were collected at 16h 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 (Muschiatti 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 well wall, like that of the pollen tube tip. Pollensome-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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