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Multilamellar bodies linked to two active plasmalemma regions in the pollen grains of *Sarcocapnos pulcherrima*

M.C. FERNÁNDEZ[1]*, M.A. PÉREZ-GUTIERREZ[2], V.N. SUAREZ-SANTIAGO[2], M.J. SALINAS-BONILLO[3], and A.T. ROMERO-GARCÍA[2]

Department of Cell Biology, Faculty of Sciences, University of Granada, E-18071 Granada, Spain[1]
Department of Botany, Faculty of Sciences, University of Granada, E-18071 Granada, Spain[2]
Department of Plant Biology and Ecology, University of Almeria, E-04071 Almeria, Spain[3]

Abstract

The presence of visible multilamellar bodies in the cytoplasm of pollen grains of at least seven species of the family *Papaveraceae* has led us to study the behaviour of these bodies during pollen-grain ontogeny and in growing pollen tubes of *Sarcocapnos pulcherrima* C. Morales & R. Garcia germinated *in vitro*. Our transmission-electron-microscope (TEM) studies in pollen grains show that the multilamellar bodies may be classified as: *1*) small, isolated and placed in the region of apertures in the cytoplasm; and *2*) large, in clusters and in contact with the active plasmalemma apertures only when tubules are being formed in the apertural intine. Similar types of multilamellar bodies to those observed in the pollen apertures can be seen near the apex of the growing pollen tube (small and isolated) and in contact with the apex plasmalemma (large and clustered). Our results support the hypothesis that the multilamellar bodies are functionally linked to moments when the cytoplasmic membrane is very active. We have also linked the multilamellar bodies to Golgi vesicles as they both react positively to acid-phosphatase (AP) staining and also to the plasmalemma by the thiocarbohydrazide-silver proteinate-staining (TCH-Sp) electron-contrasting technique.

Additional key words: ontogeny, *Papaveraceae,* pollen apertures, pollen tubes, TEM, ultrastructure.

Introduction

Intracellular membrane inclusions have been frequently described. Due to the numerous locations and conditions in which these structures may be found, they have received various names and have been endowed with diverse functions.

In plant cells, multivesicular bodies have been associated with the proteolytic processes in *Arabidopsis* (Otegui *et al.* 2006) and the protein bodies of mung bean cotyledons (Van der Wilden *et al.* 1980). Arabino-galactan proteins have been found in *Nicotiana tabacum* both in plasmalemmasomes and in multi-lamellar bodies in the pollen tubes as well as in other sub-cellular sites (Ferguson *et al.* 1999).

We describe here multilamellar bodies observed in the vicinity of apertures in the pollen grains of seven species of *Papaveraceae* and also within the apex of the pollen tube in S*arcocapnos pulcherrima*, germinated *in vitro*. We also found lamellar structures in the form of myelin bodies in *Sarcocapnos pulcherrima* pollen tubes. The myelin and multilamellar bodies described here are not related either in their structure or location.

Pollen apertures play a crucial role in the sexual reproduction of plants and have a very complex structure which undergoes significant changes during its onto-genesis (Fernández and Rodríguez-García 1989, 1995). One of the clearest apertural changes observed in some taxa is the differentiation of the apertural intine, involving a lens-shaped thickening known as the intinous oncus which plays an important role in germination. The cytoplasmic membrane below the intinous oncus is very

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Abbreviations: AP - acid-phosphatase; TCH-Sp - thiocarbohydrazide-silver proteinate; TEM - transmission electron microscopy.
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* Author for correspondence: fax: (+ 34) 958 243258, e-mail: mcfdez@ugr.es

active producing tubules that extend towards the intine and generate a layer of membranous tubules in the intinous oncus. Initially, the tubules are in contact with the plasmalemma and finally they separate from the cytoplasm membrane (Rodríguez-García and Fernández 1988, Fernández *et al.* 1992).

Another very active area of the cytoplasmic membrane is the apical zone of the pollen tube. This is one of the areas of rapid growth in eukaryotic cells and the cytoplasmic membrane of this zone is very active during exocytosis and endocytosis, although the sub-cellular location of this growth is as yet unknown. Zonia and Munnik (2008) found that the apex is the area where endocytosis and exocytosis occur but that the membrane surface area also increases rapidly.

The aim of our study was to detect multilamellar bodies in the pollen grains of seven species of the *Papaveraceae* family and in the pollen tubes of *Sarcocapnos pulcherrima* using transmission electron microscope and to classify them according to their size and location. We used acid-phosphatase (AP) staining (Megías and Renau 1998) to establish a possible relationship between the multilamellar bodies and other neighbouring cytoplasmic organelles. To determine the relationships between the plasmalemma and multi-lamellar bodies we also used thiocarbohydrazide-silver proteinate-staining (TCH-Sp), an electron-contrasting technique specific to the plasma membrane of plant cells (Weber 1992).

Materials and methods

Developing anthers and fresh pollen were collected from: *Sarcocapnos pulcherrima* C. Morales & R. Garcia (subfamily *Fumarioideae*, tribe *Fumarieae*, wild population sample number GDAC 22851); *Hypecoum imberbe* Sm. (subfamily *Hypecoideae*, wild population sample number GDAC 22824; *Fumaria capreolata* L. (subfamily *Fumarioideae*, tribe *Fumarieae*, wild population sample number GDAC 22941; *Platystemon californicum* Benth. (subfamily *Papaveroideae*, tribe *Platystemonoideae*, living collection accession number 20051151-87, National Botanical Garden of Belgium); *Pseudofumaria alba* (Mill.) Lidén (subfamily *Fumarioideae*, tribe *Fumarieae*, living collection accession number 1985-0760, Botanical Garden of Göteborg, Sweden; *Euptelea poliandra* Siebold & Zucc (family *Eupteleaceae*, (living collection accession number 19723330, National Botanical Garden of Belgium; and *Pteridophyllum racemosum* (subfamily *Pteridophylloideae*, living collection accession number 1982-0246, Botanical Garden of Göteborg, Sweden).

Anthers of the 7 species in different phases of pollen maturation were collected. Samples were prefixed in 3 % (m/v) glutaraldehyde with 0.025 M cacodylate sodium buffer (pH 7.2) at room temperature for 24 h. They were washed with several changes of cacodylate buffer and post-fixed in 1 % (m/v) OsO_4 for 2 h. They were then dehydrated in a graded series of ethanol and embedded in *Epon*. Ultra-thin sections were cut on a *Ultracut E* (*Leica Microsystems*, Wetzlar, Germany) and stained with 2 % (m/v) uranyl acetate and lead citrate (Reynolds 1963). Observations were carried out with a *Carl Zeiss* (Jena Germany) *LIBRA 120 Plus* transmission electron microscope (TEM).

For AP cytochemistry conducted in *Sarcocapnos pulcherrima*, we followed Megías and Renau's (1998) procedure using samples without post-fixation in osmium tetroxide. For TCH-Sp cytochemistry in osmium-fixed material from *Sarcocapnos pulcherrima*, we followed Weber's procedure (1992).

For *in vitro* germination, pollen grains of *Sarcocapnos pulcherrima* were kept at 25 ºC in Brewbaker and Kwack's medium (1963) and fixed after 1 and 2 h incubation and germination of the pollen tube.

Results

The multilamellar bodies found in the pollen of the seven species studied were electron dense, irregular in shape, and dispersed within the cytoplasm, although clearly within the region of the pollen wall and most frequently below the apertural zones (Fig. 1*A*). They appeared only from the intermediate bicellular stage during pollen ontogeny (Fig. 1), when the generative cell was embedded within the pollen grain, until maturity. During the intermediate bicellular stage, the intine of the apertural areas thickens into a lens shape forming the intinous oncus within which a large number of tubules either in the process of formation or growth were seen (Fig. 1*A*). The cytoplasmic membrane in the areas below the oncus was very active producing a large number of growing tubules that extend towards the intine (Fig. 1*B*). The tubules were formed from the plasmalemma and groups of large multilamellar bodies could be seen in contact with it (Fig. 1*B*). The multilamellar bodies were composed of various peripheral membranous layers arranged more or less compactly around an apparently empty electron-transparent core (compare Fig. 2*A*,*B*,C,*D*, and *E*). Some of the multilamellar bodies contained only compact lamellars and sometimes had a membranous edge (Fig. 2*C*).

After *Sarcocapnos pulcherrima* pollen grains were treated by TCH-Sp staining, the multilamellar bodies

appearred to be opaque with much deeper staining than the cytoplasm itself (Fig. 3*A*,*B*). In mature pollen, the size of the multilamellar bodies and their degree of association depended upon their proximity to the apertural zone. It can be seen in Fig. 3*A* that there are small, isolated multilamellar bodies dispersed throughout the cytoplasm whilst groups of large multilamellar bodies can be seen in the region of the apertural zone and in contact with the plasmalemma. It is noteworthy that the multilamellar bodies in contact with the plasmalemma are on both sides of the membrane (3*A*). They varied from quite small vesicles of less than 0.2 μm, which were normally discrete from one another, to large multilamellar bodies of 0.2 - 1.2 μm, which tended to appear clustered together in groups. Both types coexisted in the same pollen grain but were distributed in different ways (Fig. 3*A*).

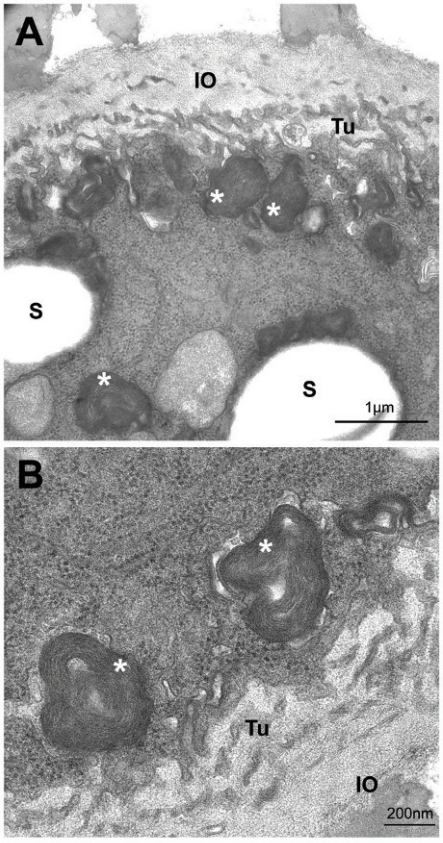

Fig. 1. Ultrastructure of the aperture and adjacent multilamellar bodies in the intermediate ontogenic stage of *Platystemon californicum* pollen. *A* - The intinous oncus can be seen with tubules (Tu) deriving from the plasmalemma. An accumulation of large multilamellar bodies (*) close to apertures and some in contact with membranes of the plasmalemma and nascent tubules. Starch grains (S). *B* - Detail of the developing aperture. The multilamellar bodies can be distinguished by their cores, the wavy plasmalemma membrane and the nascent tubules (Tu). IO - intinous oncus.

The plasmalemmas of the pollen grains were also specifically stained with TCH-Sp appearing as a fine electron-opaque sinuous lining beneath the pollen wall. Some multilamellar bodies close to the plasmalemma were also stained (Fig. 3*B*).

The multilamellar bodies in the pollen grains proved positive to AP as shown by their greater electron-density compared to other nearby organelles such as dictyosomes (Figs 4*C*). Nevertheless, some Golgi vesicles turned out to be just as AP-positive as the multilamellar bodies (Fig. 4*C*).

Multilamellar bodies similar to those described in the pollen were observed in the pollen tubes after at least 2-h growth in the germination medium. Small, isolated multilamellar bodies continued to be seen in areas of the cytoplasm adjacent to the pollen wall. In pollen-tube slices taken from the vicinity of the apex, small multilamellar bodies can be seen dispersed in the cytoplasm near the wall and large ones in contact with the cytoplasmic membrane (Fig. 4*A*). The cell walls in the area of the growing pollen-tube apex are made up of just one fibrillar layer (Fig. 4*A*).

In slices taken from areas of the pollen tube at a greater distance from the apex, there are separate myelin figures. These contain loosely arranged, peripheral membranous layers and are normally associated with various membranous vesicles within a vacuole covered with thin bands of cytoplasm (Fig. 4*B*). It can be seen that the myelin bodies are not in contact with the cytoplasmic membrane but are located more internally. The cell walls in these areas at some distance from the growing apex of the pollen tube are made up of two layers (Fig. 4*B*).

Discussion

Our detailed study has revealed the existence of cytoplasmic multilamellar structures in pollen in some of the taxa of the *Papaveraceae* family. These structures appear most widely in the *Fumaroideae* subfamily although they have also been detected in the *Papaveroideae* and *Hypecoidea* subfamilies and in one member of the *Eupteleaceae* family. The fact that these structures appear in this latter family, considered to be at the base of the order *Ranunculales* and in *Hypecoidea*, which is the basal clade of the *Papaveraceae* family (Hoot *et al.* 1997) as well as in taxa of the two most important subfamilies, may indicate that these structures are widespread throughout the whole group.

It is well known that pollen grains accumulate large quantities of intracellular reserves and extensive membranous labyrinths (McCoy and Knox 1988, Dinis and Mesquita 1999). The polarity observed in the distribution of the multilamellar bodies in *Papaveraceae* pollens supports the idea that practically all the structures and organelles in the pollen and tubes have polarity (Rodríguez-García and Fernández 1990, Rodríguez-García *et al.* 2003). The reason for the accumulation and

polarity of the multilamellar bodies described in this study can be understood in the light of when and where they appear: firstly, during the formation of the apertural intinous oncus, and secondly, during the initial apical growth of the pollen tube.

Many types of pollen from various taxonomic groups, among which those studied in this paper belong, show a lens-type thickening known as intinous oncus. In some members of the *Fumariaceae* family, the intinous oncus appears to be well developed whilst less so in others (Romero and Fernández 2000, Romero *et al.* 2003). The intinous oncus undergoes a ripening process during pollen ontogeny (Rodríguez-García and Fernández 1988, Fernández and Rodríguez-García 1989, 1995, Fernández *et al.* 1992), becoming filled with tubules formed from invaginations in the cytoplasmic membrane which in maturity separate from the plasmalemma. The tubules finally become full of proteins of gametophytic origin and play a role in pollen stigma recognition. One further function of the intinous oncus, when the tubules are in contact with the plasmalemma and empty of protein, is to allow the grain to communicate with the outside (Fernández and Rodríguez-García 1990) at the critical moment when it is at maximum metabolic activity and requires diverse communication mechanisms (Dinis and Mesquita 2004). The intinous oncus is very complex both structurally and functionally and the multilamellar bodies in contact with its plasmalemma, which are described for the first time in this paper, add further complexity and interest. As far as we know, endocytosis processes are not

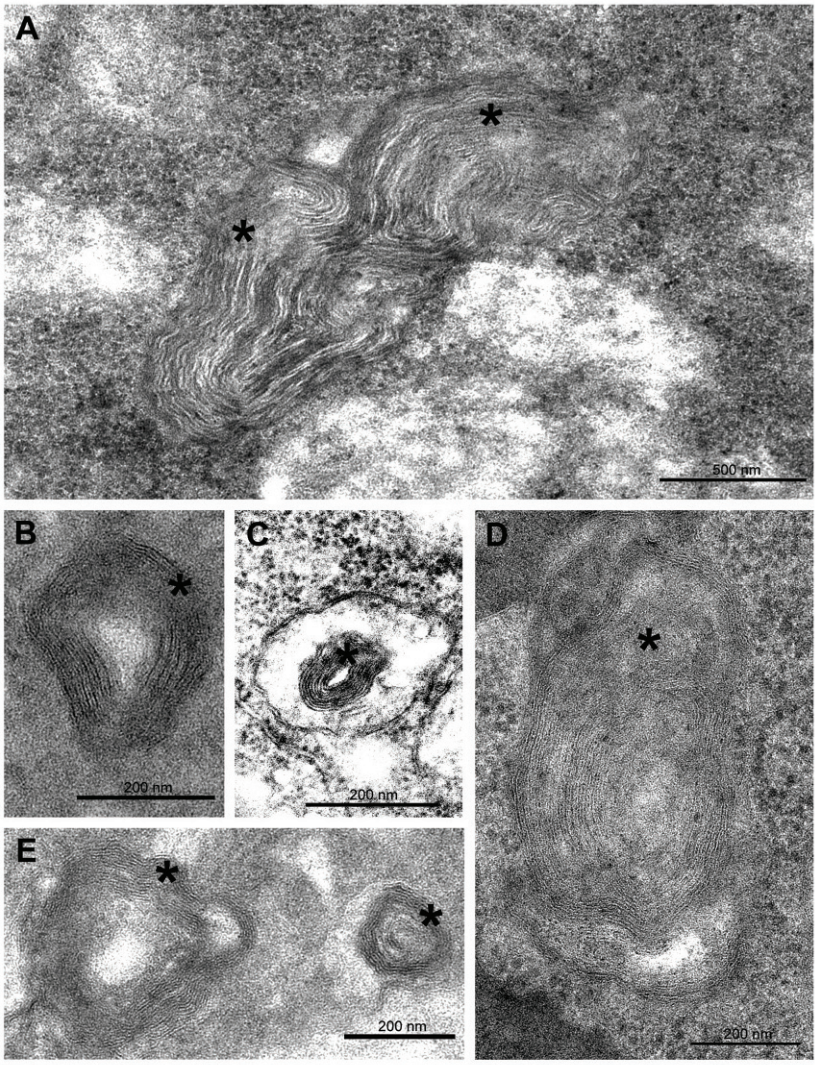

Fig. 2. Multilamellar cytoplasmic bodies in different *Papaveraceae* pollen species. *A* - Two multilamellar bodies in *Fumaria capreolata*; irregularly shaped, electron-dense structures with various layers of compact peripheral membranes around a core. *B* - *Pteridophyllum racemosum*; peripheral, membranous layers around an electron-transparent core. *C* - *Hypecoum imberbe*; loosely associated peripheral membranous layers around an electron-transparent core and a membranous edge made up of a double membrane. *D* - *Platistemon californicum*; multilamellar body of irregular shape containing compact membranous layers and a slightly electron-dense core. *E* - Multilamellar bodies observed in *Sarcocapnos pulcherrima;* compact peripheral membranous layers with an electron-transparent core, * - multilamellar bodies.

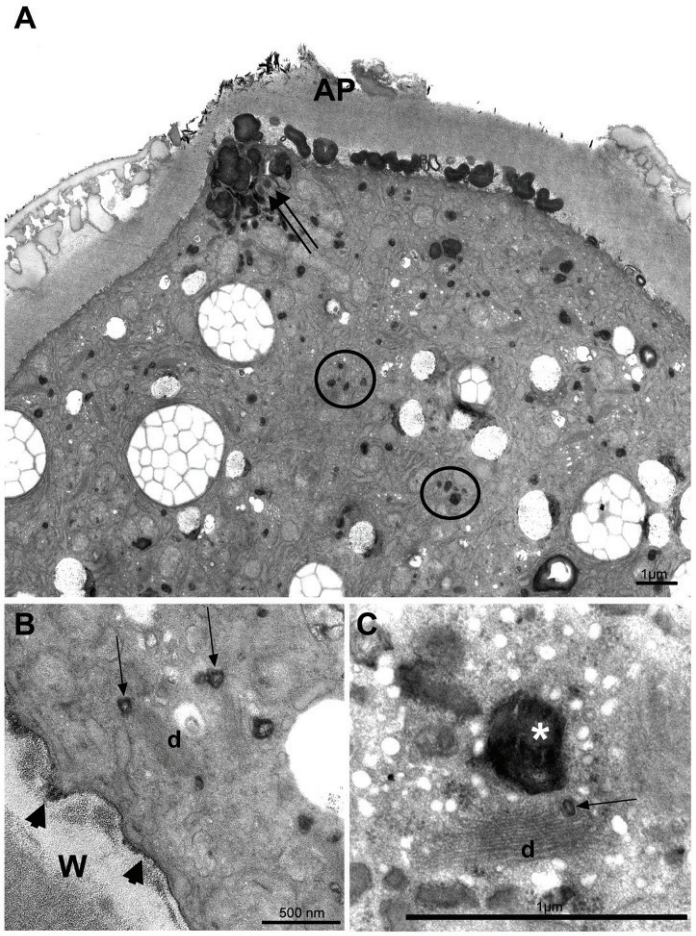

Fig. 3. Cytochemistry in mature pollen of *Sarcocapnos pulcherrima*. *A* - TCH-Sp in apertural zone (AP). In the cytoplasm below the aperture there are both large (*double arrow*) and small isolated (*circles*) multilamellar bodies. The large ones are interconnected and also in contact with the plasmalemma. *B* - TCH-Sp. Positive reaction to intracytoplasmic multilamellar bodies (*arrows*) and plasmalemma (*arrow heads*) can be distinguished (W - pollen wall, d - dictyosome). *C* - AP-positive multilamellar bodies (*) associated with dictyosomes (d) and Golgi vesicles. Note that some small membrane vesicles close to dictyosomes are AP-positive (*arrow*).

known to occur in the plasmalemma of the aperture and so we suggest that multilamellar bodies are involved in the formation of tubules to store and release lipids at the opportune moment as "membrane exporters". Multilamellar bodies are membranous bodies that contain mainly unsaturated lipids similar to the plasmalemma as it is demonstrated after treatment with TCH-Sp (Weber 1992).

Although Ferguson *et al.* (1999) did not carry out an ultra-structural study on the multilamellar bodies, the images published in their paper show bodies in pollen and tubes of similar shape and size to those we have described here. The pollen tube grows extremely rapidly as far as eukaryotic cells are concerned. This growth is supported in the apical zones by high exocytosis and endocytosis which return the fluid phase and membrane to the interior. Both processes seem to be vital for the rapid growth of systems such as the pollen tube (Lisboa *et al.* 2008). In their experiments to ascertain the movement of vesicles in the pollen tubes of *Nicotiana tabacum*, Zonia and Munnik (2008) demonstrated that the apex is the area where endocytosis and the reconversion of membranes occur, whilst exocytosis takes place in the immediately adjacent areas. Recently, a new type of organelle, EXPO, has been described which has two membranes and fuses with the plasma membrane of the vegetal cell (Wang *et al.* 2010). The EXPO is described as being an exocyst-positive organelle, different from endosomes and autophagosomes, that mediates cytosol to cell-wall exocytosis. It is clear that exocytosis and endocytosis coexist in the pollen-tube apex. We might hypothesize that the multilamellar bodies of *Sarcocapnos* are involved in pollen-tube growth but we cannot determine whether they are involved in exocytosis or endocytosis. In the light of the complete ultrastructural similarity of the multi-lamellar bodies that appear in the apertures and pollen-tube apex, we may deduce that they both have similar functions. Mainly on the basis of this similarity, we agree with the idea that they are also related in some way with increasing the bulk of the membrane.

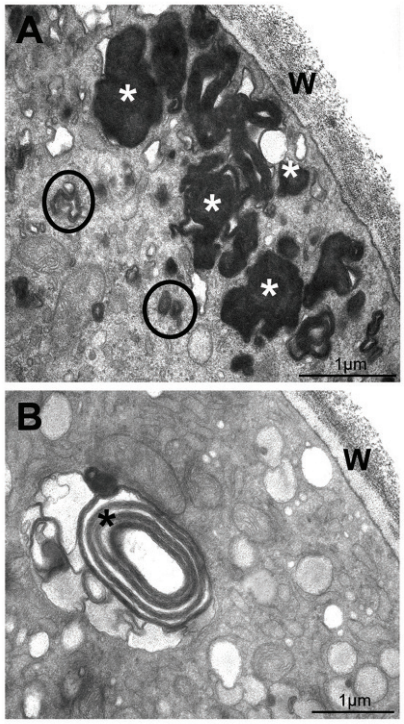

Fig. 4. Sections of pollen tube from *Sarcocapnos pulcherrima*. *A* - TCH-Sp. Zone close to the growing apex. There are both large (*) and small (*circles*) multilamellar bodies within the cytoplasm beneath the wall of the tube formed by a single fibrillar layer (W). It can be seen how the large, irregularly shaped bodies are interrelated and close to or in contact with the plasmalemma. The small bodies are to be seen deeper within the cytoplasm and are not interconnected. *B* - A myelin figure (*) in an area some way removed from the apex with a bilayered wall (W). Its membranous layers are quite loose with cytoplasmic bands between them; they are also associated with membranous vesicles.

We also found separate myelin figures in areas at some distance from the growing apex and in deep intracytoplasmic zones in *Sarcocapnos pulcherrima* pollen tubes. Robards and Kidwai (1969) put forward the idea that myelin bodies play an important role in the transport of precursors and enzymes away from the plasmalemma while cells are adapting to adverse conditions. Low temperatures, for example, induce ultrastructural changes among which the appearance of myelin structures in epidermal and mesophilic leaf cells (Kratsch and Wise 2000, Stefanowska *et al.* 2002) and in root cells (Glinska *et al.* 2009) happen.

We are as sure as we can be that the pollen grains with multilamellar bodies described in this work are not artefacts due to stress caused in plants growing in areas polluted by heavy metals, such as those described by Dinis and Mesquita (2004) and Pandey *et al.* (2009), for example. The anthers of our species came from very different origins (see Materials and methods) and new species of the *Papaveraceae* family with similar ultrastructural bodies are constantly being discovered. To discern these bodies clearly within a cytoplasm dense in other structures and organelles, it is essential, as we have done in this study, to be able to apply cytochemical techniques such as TCH-Sp which specifically stain plant membranes (Weber 1992).

In summary, we describe here for the first time multilamellar bodies linked to active plasmalemma in pollen apertures. We also describe similar bodies in pollen-tube apexes. The main role that we propose for these bodies is that of "membrane exporters" in the zones where the membrane is actively increasing in surface, apertures during intinous oncus formation, and pollen-tube apexes. Further proof of this interpretation will be to locate membrane markers of the exocytosis process, such as the exocyst protein Exo 70, within these bodies (Wang *et al.* 2010).

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