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Ultrastructural study of the relationship between generative and vegetative cells in *Magnolia* × *soulangeana* Soul.-Bod. pollen grains

A. M. Dinis* and **J. F. Mesquita**

Laboratory of Electron Microscopy and Phycology, Department of Botany, University of Coimbra, Coimbra

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Summary. In *Magnolia* × *soulangeana* pollen grains the generative cell (GC) does not become totally free within the vegetative cell (VC), at least until the pollen tube emergence. Due to a deviation in its detachment process from the sporoderm, the opposing ends of the VC plasmalemma do not fuse themselves when the GC moves away from the intine. Consequently, the interplasmalemmic space surrounding the GC does not become isolated but rather maintains continuity with the sporoderm through a complex formation that we have called plasmalemmic cord. The real existence of this formation was confirmed through serial sectioning showing the plasmalemmic cord to consist of the VC plasmalemma. In its initial portion it is occupied by a reasonably accentuated wall ingrowth of the inner layer of the intine (intine 3). In the remainder portion, neither of the cytochemical tests used in this work have revealed the presence of a significant amount of wall material. However, ultrathin sections of samples processed either chemically or by cryofixation showed the existence of an intricate system of tubules and vesicles, some of which are evaginations of the VC plasmalemma. The hypothesis that the plasmalemmic cord may have a role in the complex interactions between the two pollen cells is discussed.

Keywords: Plasmalemmic cord; Pollen grain; Ultrastructure; *Magnolia* × *soulangeana*.

Introduction

The first pollen mitosis is a highly asymmetric division that results in the production of two unequal daughter cells, the vegetative (VC) and generative cells (GC). These have distinct cytoplasmic constitution and different developmental fates. The GC divides to form the pair of sperm cells. The VC elongates to form the pollen tube that grows towards the embryo sac, carrying the sperm cells. The regulatory mechanisms controlling the differential behavior of both the pollen cells are still unclear (for reviews, see McCormick 1993, Bedinger et al. 1994, Tanaka 1997).

When first formed the GC is appressed to the pollen wall as a "meniscus". Later on, the GC becomes detached from the sporoderm and moves towards the vegetative nucleus, both establishing a close physical association (Mogensen 1992). The detachment process, often compared with the formation of a drop of water, seems to be rather constant among the different species leading to the complete engulfment of the GC by the VC (for reviews, see Sunderland and Huang 1987, Schlag and Hesse 1992). Thus, the GC will become a free cell within the VC cytoplasm. Recent studies indicate that complex interactions are established between the two pollen cells (Tiwari 1994) and that a functional VC is needed for the correct development and function of the GC (Twell 1995). How these interactions are established is poorly understood.

During our studies on the development of *Magnolia* × *soulangeana* pollen grains (e.g., Dinis and Mesquita 1992, 1994a, b; Dinis 1997), we found that the GC differentiation is different from that traditionally accepted for most of the pollen grains. Instead of attaining the typical suspended state in the VC cytoplasm, the GC remains somewhat connected with the sporoderm through a complex formation that we have called plasmalemmic cord. In this paper we describe

*Correspondence and reprints: Laboratory of Electron Microscopy and Phycology, Department of Botany, University of Coimbra, P-3049 Coimbra Codex, Portugal.
E-mail: amdinis@ci.uc.pt

the evolution of the GC in its early phase of development, with particular emphasis on the characterization of the plasmalemmic cord.

Material and methods

Plant material, pollen staging, and pollen plasmolysis

Anthers of *Magnolia* × *soulangeana* Soul.-Bod. from the Coimbra Botanical Garden (Coimbra, Portugal) were used in this study. The stage of pollen development (see characterization in Dinis and Mesquita 1994a) was checked in light-microscopic (LM) observations of either acetocarmine squashes or semithin sections of material processed for transmission electron microscopy (TEM) and stained according to Humphrey and Pittman (1974). Plasmolysis of some pollen grains was intentionally induced by suspending them in a 30% solution of sucrose.

Electron microscopy

Conventional TEM fixation. Samples of anthers were fixed for 2–4 h, at room temperature, in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.1, supplemented with 1 mM calcium chloride. Following rinsing in the same buffer, samples were postfixed for 1 h in 1% buffered tetroxide osmium, rinsed again, dehydrated in a graded ethanol series, and embedded in low-viscosity resin (Spurr 1969).

Freeze-fixation and freeze-substitution. Samples of anthers containing pollen at an intermediate stage of maturation were rapidly plunge frozen in liquid nitrogen slush that was obtained using a Polaron CryoTrans model E7450. They were then transferred in liquid nitrogen to small vials containing 2% osmium tetroxide in anhydrous acetone precooled to –80 °C (Lancelle et al. 1986). Substitution was carried out for approximately 48 h, after which the vials were gradually warmed to room temperature over a period of approximately 24 h. After rinsing in acetone and then methanol, the samples were en bloc stained with 5% uranyl acetate in methanol for 2 h, rinsed in pure methanol and infiltrated and embedded in Spurr's resin.

For both fixations, thin sections were obtained with an ultramicrotome LKB Ultrotome NOVA equipped with a diamond knife, conventionally stained with uranyl acetate and lead citrate, or with lead citrate only, and observed in a JEOL JEM-100 SX at 80 kV.

Cytochemistry

Detection of callose was performed in fresh anthers that were immersed in a 0.01% solution of aniline blue in 0.15 M K_2HPO_4, pH 9.2, during 15 min (Currier and Strugger 1956). Pollen grains were further released from anthers by gentle pressure and examined under ultraviolet irradiation in an epifluorescence microscope (Nikon Optiphot model XF-EF). For control purposes, pollen grains of anthers that have been immersed in distilled water were observed.

To detect insoluble polysaccharides with the LM, 2 µm thick sections were subjected to the periodic acid-Schiff reaction according to the procedure of Jensen (1962). For control purposes, sections in which the periodic acid oxidation was omitted were observed.

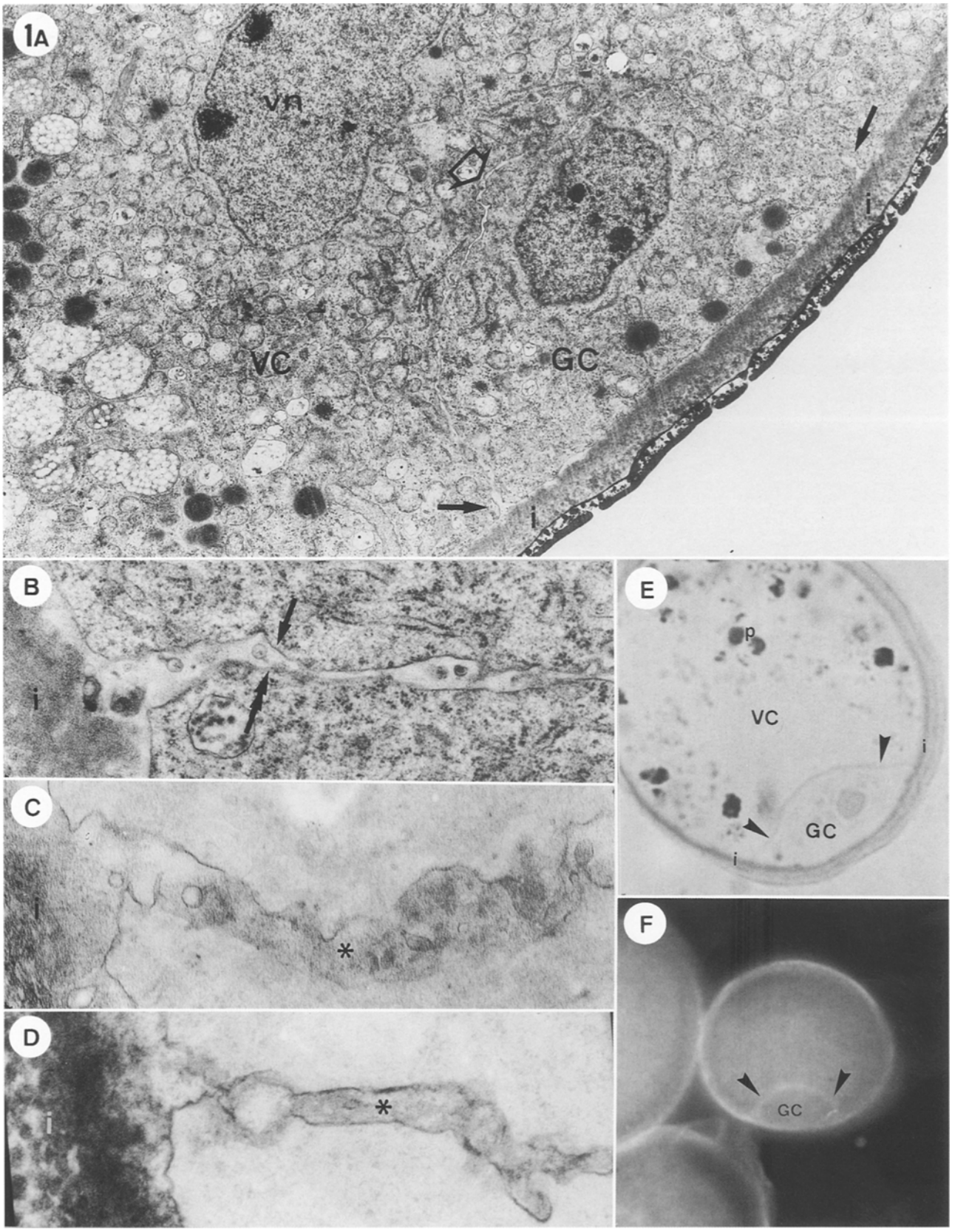
To detect neutral polysaccharides and other carbohydrates with the TEM, ultrathin sections were collected on gold grids and floated for 15 min on 10% aqueous solution of hydrogen peroxide at room temperature. Following washing in distilled water, the grids were treated with periodic acid-thiocarbohydrazide-silver proteinate according to

Fig. 1 A–F. Young pollen grains of *M.* × *soulangeana* just after the generative cell (*GC*) formation. **A** General view showing the periplasmic space (open arrow) surrounding the GC and its attachment sites (arrows) to the intine (*i*). *VC* Vegetative cell; *vn* vegetative nucleus. × 6,250. **B** Detail of the GC periplasm in the junction zone with the intine (*i*). Numerous inclusions are seen in the space between the VC plasmalemma (single arrow) and the GC plasmalemma (double arrow). × 30,000. **C** and **D** Ultrathin sections of the same zone shown in **B** after, respectively, the Thiery test and the PTA staining at low pH. A positive staining is seen both in the intine (*i*) and in the GC periplasm (asterisk). × 30,000. **E** Semithin section after the periodic acid-Schiff reaction. Like the intine (*i*) and the amyloplasts (*p*) in the VC, the GC periplasm (arrowheads) is positively stained. × 1,750. **F** Young pollen grains stained with aniline blue. An arched fluorescent layer (arrowheads) surrounds one side of the GC. The pollen wall shows autofluorescence as checked in the control experiment (not shown). × 580

Fig. 2 A, B. Pollen grains of *M.* × *soulangeana* during the GC migration to a position more deeply inside the VC. **A** Detail of the junction zone of the GC periplasm with the intine (*i*), showing accumulation of amorphous material (asterisk) together with several inclusions. × 30,000. **B** Bulblike appearance of the GC in a slightly older stage as that in **A**. Numerous lipid droplets (arrows) accumulate in the VC cytoplasm lining the GC, while the amorphous material (asterisk) is now distributed over the whole original attachment area of the GC to the intine (*i*). × 6,000

Fig. 3 A–E. Intermediate pollen grains of *M.* × *soulangeana*. **A** Median thin section showing the spherical GC surrounded by a collar of lipid droplets, and the original attachment area (arrows) of the GC to the intine. A portion of the plasmalemmic cord (arrowhead) is also evident. *p* Amyloplast. × 4,800. **B** and **C** Thin sections of the zone where the GC was initially formed after, respectively, the Thiery test and the PTA staining at low pH. A negatively stained layer (asterisk) is seen between intine 2 (i_2) and the newly formed intine 3 (i_3) (compare with **A**). i_1 Intine 1; *e* exine; *p* amyloplast; *l* lipid droplet. × 30,000. **D** and **E** GC periplasm (arrow) after, respectively, the Thiery test and the PTA staining at low pH. *l* Lipid droplets. × 25,000 and × 30,000, respectively

Fig. 4 A–F. Ultrastructure and cytochemistry of the plasmalemmic cord in intermediate pollen grains of *M.* × *soulangeana*. **A** Portion of a pollen grain showing several membranous structures (arrows) near the VC nucleus (*vn*) and surrounded by numerous lipid droplets (*l*). *p* Amyloplast. × 9,600. **B** Higher magnification of the membranous structure on the right in **A**. Note the numerous vesicular and tubular profiles, some of which are tubular evaginations (arrowheads) of the VC plasmalemma. × 40,000. **C** Appearance of a membranous structure after the Thiery test. Arrowheads point to tubular evaginations of the VC plasmalemma. *l* Lipid droplets. × 30,000. **D** Sections of the plasmalemmic cord (arrows) after PTA staining at low pH. One of the sections (arrowhead) is in continuity with the GC periplasm. *l* Lipid droplets. × 33,000. **E** Portion of the plasmalemmic cord (arrow) in the junction zone with the GC periplasm. × 44,000. **F** Portion of the plasmalemmic cord in the junction zone with the sporoderm (*e* exine; i_1 intine 1; i_2 intine 2; i_3 intine 3). The arrows show that the unit membrane of the plasmalemmic cord is the VC plasmalemma. PTA staining at low pH. × 6,000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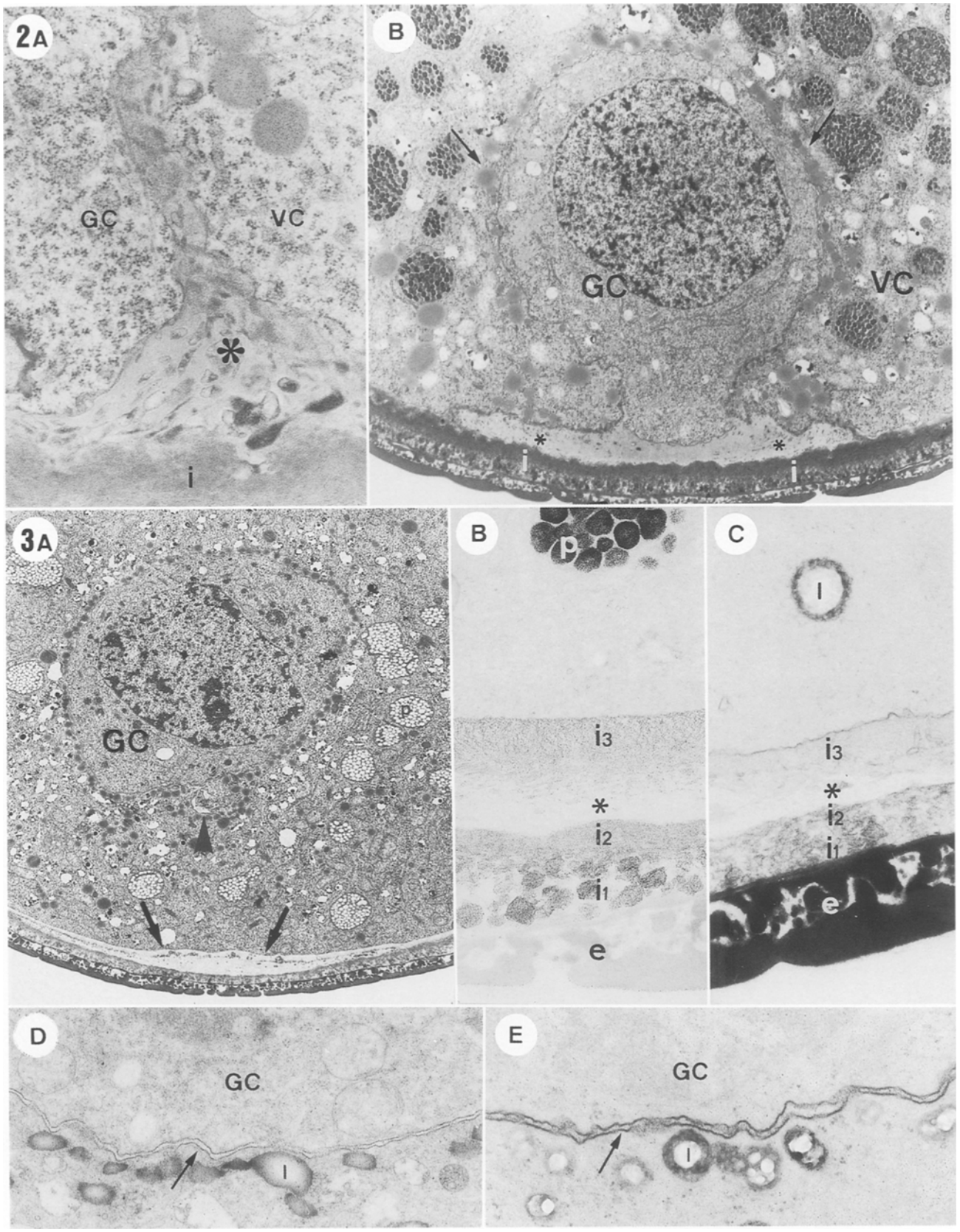
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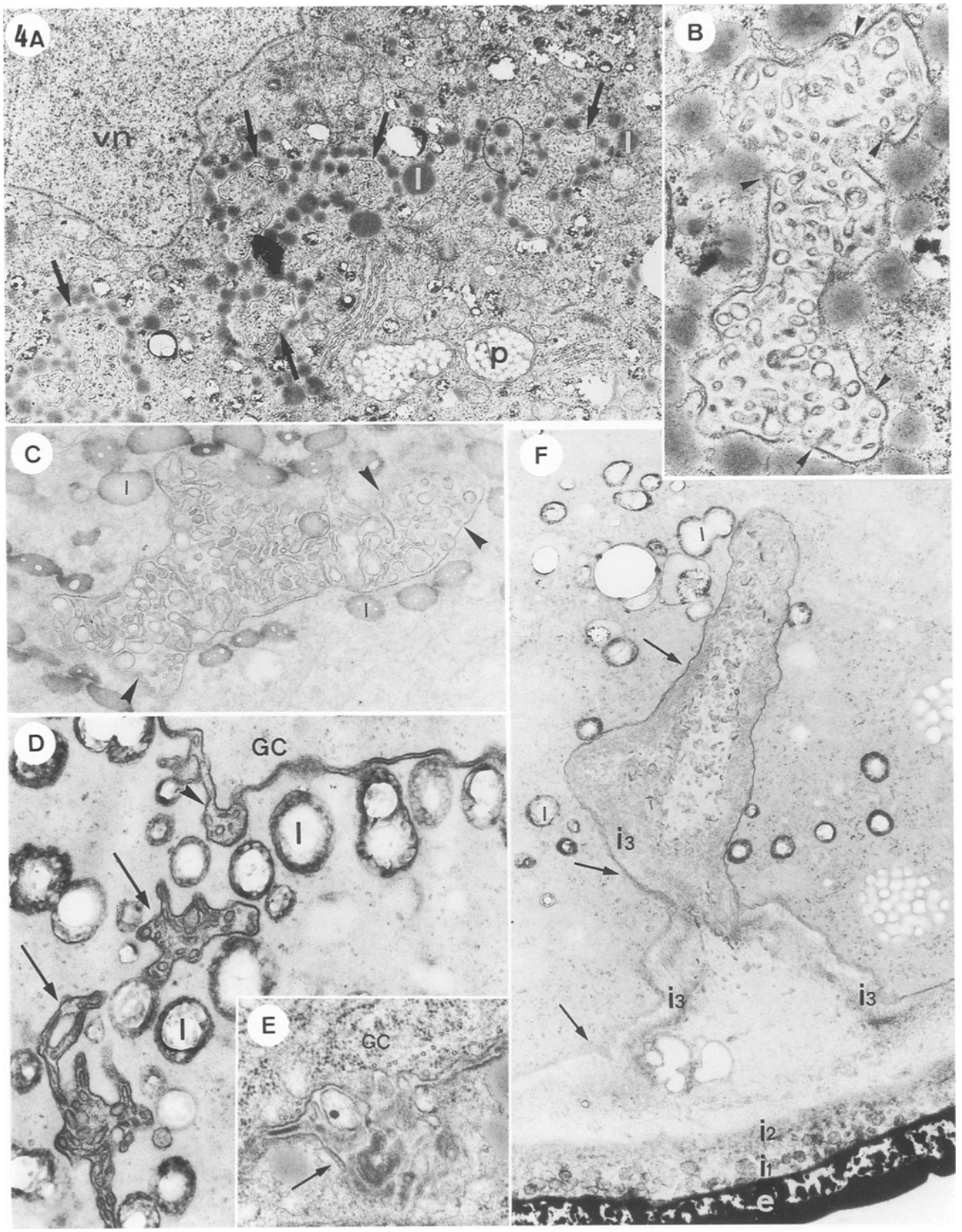
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the method of Thiery (1967). For control purposes, sections were treated in the same way but without periodic acid oxidation.

Acidic polysaccharides were detected with the TEM by the use of phosphotungstic acid (PTA) at low pH (Rambourg 1969, Farragiana and Marinozzi 1979). After osmium has been removed from the sections, they were stained with a 1% solution of PTA in 10% chromic acid. For control purposes, sections not treated with the PTA solution were observed.

Reconstruction of the plasmalemmic cord

Serial sections with a thickness of approximately 500 nm were collected and mounted on formvar-coated slot grids according to Galey and Nilsson (1966). After being stained with uranyl acetate and lead citrate, the serial sections were photographed at the TEM at 100 kV, and printed at the same magnification. Transparent acetate sheets were then placed on the photographic prints of each section to produce tracings of the membranous structures belonging to the plasmalemmic cord, together with the outlines of the GC nucleus, the GC–VC interface, and the different layers of the pollen wall. The latter were taken as marks to produce the correct alignment of the transparent acetate sheets when these were superimposed to be photographed in the vertical axis (corresponding to the incidence of the electron beam on the sections).

Results

In a previous work we have described the nuclear and cytoplasmic evolution of the GC during pollen maturation (Dinis and Mesquita 1994a). Therefore, these features will not be included here, and the present work focuses mainly on the characteristics of the GC–VC interface.

The newly formed GC was lenticularly shaped and attached to the intine (Fig. 1 A). Usually, it was located on the side opposite the sulcus lying reasonably close to the VC nucleus (Fig. 1 A). The GC and VC were separated by their respective plasma membranes and these, in turn, were separated by an electron-transparent space (periplasm) where vesicular profiles and a few inclusions with varying electron density could be seen (Fig. 1 B). Following the Thiery test (Fig. 1 C) and the PTA staining at low pH (Fig. 1 D), material was detected in the periplasm that reacted similarly as the intine, with which it was contiguous. Like the intine and the amyloplasts in the VC cytoplasm, it also stained positively after the periodic acid-Schiff reaction allowing the clear distinction of the GC outline (Fig. 1 E). Following staining with aniline blue it fluoresced brightly (Fig. 1 F) indicating the presence of callosic material.

When the GC began to move away from the intine, striking changes occurred in its periplasm. At first, an amorphous material with moderate electron density, and containing numerous inclusions, accumulated in the zone where the GC periplasm meets the intine (Fig. 2 A). Then, a constriction was formed in this zone and the area of the GC that was touching the intine became gradually reduced, giving the GC a bulblike appearance (Fig. 2 B). At this time numerous lipid droplets accumulated in the VC cytoplasm lining the GC periplasm and this was more reduced and uniform in thickness. Otherwise, the above referred amorphous material has now been distributed over the entire original attachment area of the GC to the intine (Fig. 2 B). As can be seen in Fig. 3 A, this material persisted in later developmental stages, always providing a useful marker for the identification of the formation site of the GC. Usually, it was located between intine 2 and the newly formed intine 3 but, in contrast, did neither react positively to the Thiery test (Fig. 3 B) nor to the PTA staining at low pH (Fig. 3 C). At this intermediate stage of maturation the GC was spheroid (15 μm in diameter; Fig. 3 A), its position being maintained close to the VC nucleus. Now, a significant amount of wall material did not exist in its reduced periplasm, as checked by the Thiery test (Fig. 3 D), the PTA staining at low pH

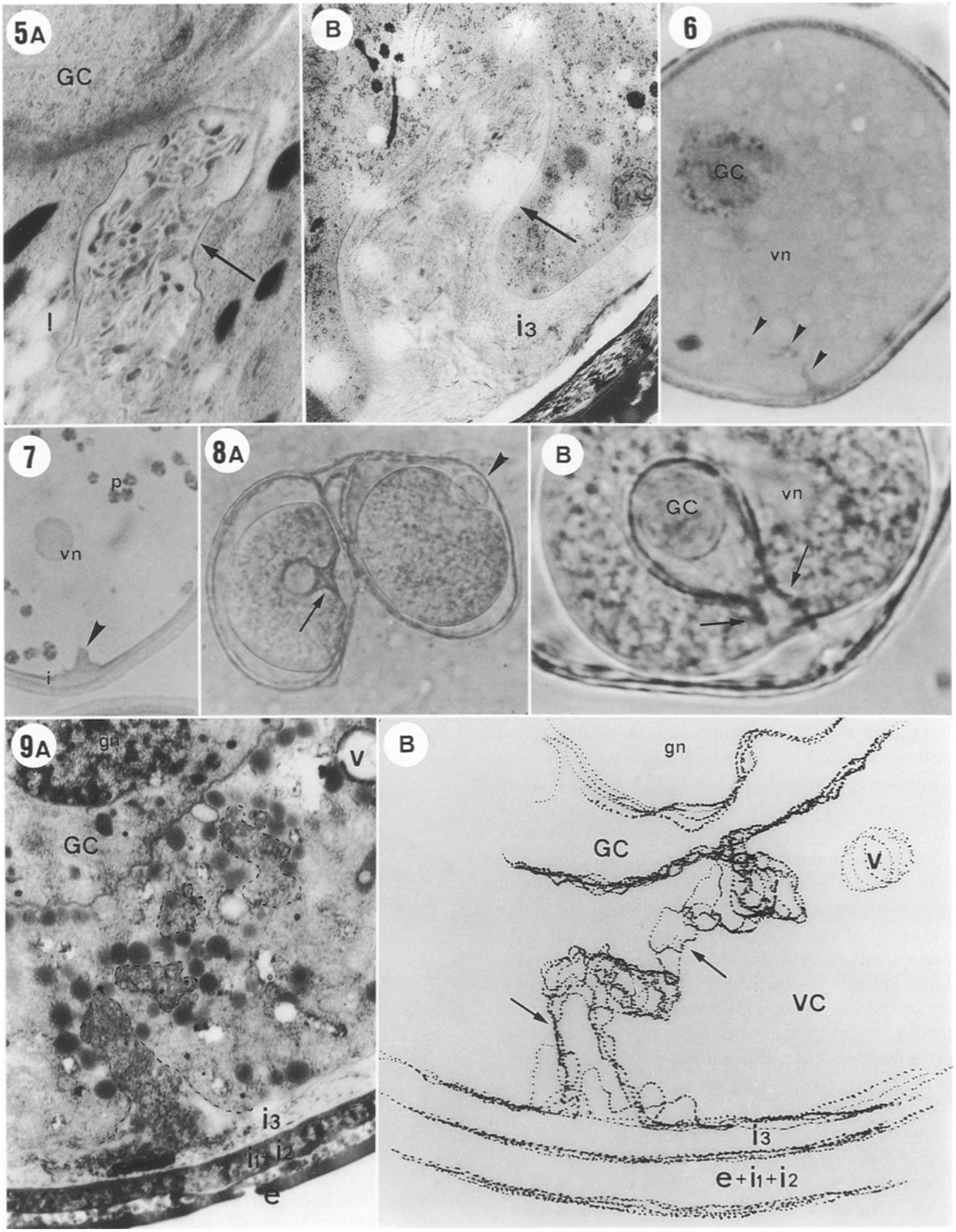
Fig. 5 A, B. Appearance of the plasmalemmic cord (arrows) after rapid freeze-fixation and freeze-substitution. **A** Portion of the plasmalemmic cord close to the GC. *l* Lipid droplet. × 44,000. **B** Portion of the plasmalemmic cord in the junction zone with the sporoderm (i_3 intine 3). × 20,000

Fig. 6. Light micrograph of a section of an intermediate pollen grain that includes the initial portion of the plasmalemmic cord (arrowheads). *GC* Generative cell; *vn* vegetative nucleus. × 1,500

Fig. 7. Part of an intermediate pollen grain after the periodic acid-Schiff reaction. Like the intine (*i*) and the amyloplasts (*p*) in the VC, the wall ingrowth occupying the initial portion of the plasmalemmic cord (arrowhead) is positively stained. *vn* Vegetative nucleus. × 1,500

Fig. 8. Plasmolysed pollen grains showing dilated plasmalemmic cords (arrows in **A** and **B**). The arrowhead in **A** points to a generative cell that left its normal position close to the VC nucleus. × 600 and × 1,500, respectively

Fig. 9 A, B. Reconstruction of the plasmalemmic cord in an intermediate pollen grain. **A** One of the seven serial sections (the dotted lines demarcate portions of the plasmalemmic cord) which were used for the reconstruction of the plasmalemmic cord (arrows in **B**). *VC* Vegetative cell; *GC* generative cell; *gn* generative nucleus; *e* exine; i_1 intine 1; i_2 intine 2; i_3 intine 3; *v* vacuole. × 13,600 and × 17,600, respectively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(Fig. 3 E), and the staining with aniline blue (not shown).

At this and later stages many sections of the pollen grains frequently showed membranous structures that, although highly variable in shape and size, were generally big enough even at low magnifications (Fig. 4 A). At higher magnification each structure appeared delimited by a single unit membrane and consisted of a complex system of tubular and vesicular profiles with varying electron density (Fig. 4 B). In favorable thin sections (see Fig. 4 B, C), at least some profiles clearly represented finger-like evaginations of the referred unit membrane, which was the VC plasmalemma (see Fig. 4 D–F). Both this unit membrane and the tubular and vesicular profiles reacted positively to the Thiery test (Fig. 4 C) and the PTA staining at low pH (Fig. 4 D, F).

Although these structures might occur individually, they usually occurred in groups, their disposition and the distance between them suggesting that they most likely were sections of the same formation (see Fig. 4 A). These and other features led us to think that this was presumably a formation linking the GC periplasm to the pollen wall, which we have named plasmalemmic cord. Effectively, the referred membranous structures always occurred either near the GC or vegetative nucleus (Figs. 3 A, 4 A, D, and 5 A), either near the original attachment area of the GC to the intine or in the region located between both (Fig. 9 A). Like the GC itself, at an intermediate stage of pollen maturation they always appeared surrounded by numerous lipid droplets (Figs. 3 A and 4 A–F). Occasionally, one of these structures was clearly seen in spatial continuity with the GC periplasm (Fig. 4 D, E) and/or with the intine of the pollen wall (Figs. 4 F, 5 B, and 9 A). In the latter case, a reasonably accentuated wall ingrowth of the inner layer of the intine (intine 3) always occupied the initial portion of the plasmalemmic cord. In addition, this wall ingrowth systematically appeared only in the site of the grain in which the GC was initially formed. It is also noteworthy that these structures had the same distribution and organization both in the chemically fixed samples and in the rapidly freeze-fixed samples (Fig. 5 A, B). This seems to exclude the possibility that they are fixation artifacts.

The probability of sectioning the whole plasmalemmic cord is very low due to its sinuous course. Figures 6 and 7 are semithin sections of pollen grains where the initial portion of the plasmalemmic cord can be seen. Figure 7 shows that the wall ingrowth occupying the initial portion of the plasmalemmic cord is positively stained after the periodic acid-Schiff reaction. Following plasmolysis of some pollen grains, the plasmalemmic cord underwent a considerable dilation becoming itself far more evident (Fig. 8 A, B). In a few cases, the GC even left its normal position close to the VC nucleus lying in the space between the pollen wall and the VC plasmalemma (Fig. 8 A). These observations together with the reconstruction of the plasmalemmic cord in an intermediate pollen grain (Fig. 9 B), which was based on seven consecutive semithin sections (of which one is shown in Fig. 9 A) clearly demonstrates that the plasmalemmic cord is a real structure that maintains the communication of the GC periplasm with the pollen wall. From the above data, Fig. 10 is our diagrammatic interpretation of the plasmalemmic cord existing in the *M.* × *soulangeana* pollen grains.

Discussion

Although in the past two decades there has been a great deal of interest in GCs, the precise nature of the GC surface is still a matter of some controversy (cf.,

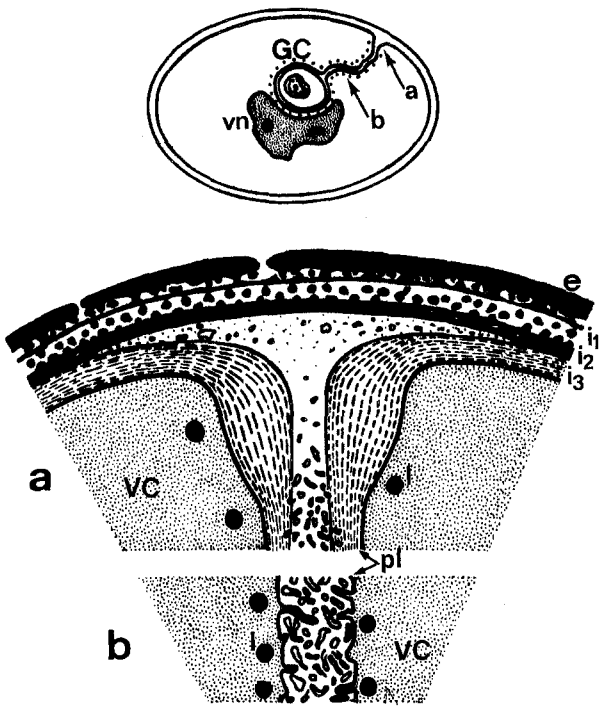

Fig. 10. Schematic representation of the plasmalemmic cord existing in a *M.* × *soulangeana* pollen grain. a and b Areas of the plasmalemmic cord identified by arrows and shown at higher magnifications. *VC* Vegetative cell; *GC* generative cell; *vn* vegetative nucleus; *e* exine; i_1 intine 1; i_2 intine 2; i_3 intine 3; *pl* vegetative cell plasmalemma exhibiting plasmatubules; *l* lipid droplets

e.g., Górska-Brylass 1970, Owens and Westmuckett 1983, Schlag and Hesse 1992, Fitzgerald et al. 1994). As has been reported for other species (Roland 1971, Albertini et al. 1981, Owens and Westmuckett 1983, Testillano et al. 1988), the cytochemical tests used in the present work reveal that the newly formed GC of *M.* × *soulangeana* has a wall consisting of callose and other polysaccharides. Later on, a true wall does not exist since part of the wall material is presumably resorbed and the other part is included in the intine in the region where the GC has initially been attached. This wall modification facilitates the deformation of the GC surface and, consequently, the GC migration to a deeper position in the VC cytoplasm together with the alteration of shape from lenticular to spherical and then spindle-shaped.

The GC differentiation in *M.* × *soulangeana* pollen grains deviates from the classical model accepted for most of the angiospermous pollen grains. Instead of becoming entirely embedded within the VC cytoplasm, the GC is somewhat connected with the sporoderm, at least until the pollen tube emergence. As far as we know, variants of the GC differentiation were only reported in the pollen tetrads of *Rhododendron* spp. (Theunis et al. 1985), in the mature bicellular pollen of *Papaver somniferum* [pers. commun. of J. M. Dunwell to Kaul et al. (1987)], and in the polyads of *Acacia retinodes* (McCoy and Knox 1988). In the last, a terminal extension of the elongated GC is linked by membrane labyrinths with the outer VC plasmalemma. These membrane labyrinths are sac-like invaginations of the VC plasmalemma that are attached to intine wall ingrowths close to the apertures of the pollen grains (see McCoy and Knox 1988). In *Rhododendron* spp., the mature GC is spindle-shaped, with two long coiled taillike extensions, of which one is connected with an intine wall ingrowth that is equidistant from the three colporate apertures (see Theunis et al. 1985). In *M.* × *soulangeana*, as in other members of the family Magnoliaceae, including *M. denudata*, *M. liliflora*, and *Michelia figo* (Dinis and Mesquita unpubl. results), the GC connection to the sporoderm is far more similar to that in *Rhododendron* spp., although with some significant differences. In either case, the origin of this connection seems to result from a modification in the detachment process of the GC. This process has been described in detail in several species and usually leads to the GC being entirely held within the VC protoplast (Schlag and Hesse 1992, and references therein). In *M.* × *soulangeana*, as presumably also in *Rhododendron* spp., the opposing ends of the VC plasmalemma do not fuse themselves when the GC moves away from the intine. Consequently, the interplasmalemmic space surrounding the GC does not become isolated but rather maintains continuity with the sporoderm through a plasmalemmic cord. Contrary to the GC connective in *Rhododendron* spp., the plasmalemmic cord in *M.* × *soulangeana* consists exclusively of the VC plasmalemma once the GC does not possess the two long coiled tails existing in *Rhododendron* spp., one of which is attached to an intine wall ingrowth. However, similarly to *Rhododendron* spp., the initial portion of the plasmalemmic cord is also occupied by a reasonably accentuated wall ingrowth of the inner layer of the intine (intine 3).

A significant feature that was not discussed by Theunis et al. (1985) is that of the complex morphologies of these connections. In *M.* × *soulangeana* both the cryofixed and the chemically fixed samples revealed that the plasmalemmic cord consists of an intricate system of fine tubular evaginations of the VC plasmalemma that may be compared to plasmatubules (cf., e.g., Harris et al. 1982, Kandasamy et al. 1988). In the germinating pollen of *Pyrus communis* Tiwari (1994) has found that wall processes were formed in the GC surface which, however, do not belong to any special connection of the GC to the sporoderm. Effectively, they can be formed on all faces of the GC and modify themselves during pollen germination. Although this does not happen in *M.* × *soulangeana*, the morphology of the wall processes in *Pyrus communis* is somewhat comparable to that of the membranous structures described in the present work, which represent sections of the plasmalemmic cord. According to Tiwari (1994) the wall processes amplify the GC surface allowing the flux of solutes between the two pollen cells, similarly to the wall ingrowths in transfer cells. Obviously, the tubular evaginations of the plasmalemmic cord also result in an enlarged plasma membrane surface that may be involved in a similar solute transport function. Theunis et al. (1985) have proposed that the GC connective in *Rhododendron* spp. would provide a life support system for the GC nutrition or biocommunication with the surface of the pollen grain. McCoy and Knox (1988) assumed that the membrane labyrinths in *Acacia retinodes* have a role in the processes of secretion involved in pollen germination. While the exact function of these GC connectives is not known from morphologic observations, a role in the complex interac-

tions between the GC and VC seems to us quite probable. As these connectives are much convoluted, it would be interesting to investigate whether they exist in other plants since they may well have gone unnoticed in earlier studies using TEM observations, or else have been disregarded as artifacts, hence their relative absence in the literature.

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