

I. Primexine development in *Passiflora racemosa* Brot.: overlooked aspects of development

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Received: 29 September 2012 / Accepted: 5 January 2013 / Published online: 14 February 2013
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Abstract Developmental stages during the tetrad period were examined in detail by transmission electron microscopy with an emphasis on substructure. Our purpose was to find out whether the sequence of sporoderm developmental events provides additional evidence for our recent hypothesis on the underlying cause of exine ontogeny as a sequence of self-assembling micellar mesophases initiated by genomically given physicochemical parameters. Osmiophilic globules encrusting the surface of postmeiotic microspores and tapetal cells are temporary prepattern units which come first. The second prepattern structures are highly ordered bundles of microfilaments and microtubules which determine the position of microspore surface invaginations and clusters of the glycolyx inside them. The first glycolyx units are microgranules which during the middle tetrad stage rearrange into radially oriented rod-like units. The latter form lens-like clusters of the glycolyx-1, located inside the invaginations. These clusters predestine the position of the future luminae in the exine reticulum. The second glycolyx layer is laid down as a continuous layer over the whole microspore surface and has similar substructure, that is radial rods. Glycolyx-2 is a framework for procolumellae which appear at the late tetrad stage. Therefore, the sequence of substructural units in the primexine is: globules, microgranules, rod-like units,

and layers of radially oriented rods tightly packed in the periplasmic space. This sequence corresponds to the first three mesophases of self-assembling micelles: spherical micelles, cylindrical micelles, and layers of hexagonally packed cylindrical micelles (middle mesophase). We observed the same sequence in other species during primexine development, and the findings of this study provide new evidence for our hypothesis.

Keywords Pollen wall and tapetum development · Pattern formation · Self-assembly · Micelles · *Passiflora*

Abbreviations

AL	Anther locus
C	Callose
Co	Columella
D	Dictyosome
ER	Endoplasmic reticulum
G	Glycolyx
G1	Glycolyx 1
G2	Glycolyx 2
GV	Golgi vesicles
LG	Lipid globule
mc, MC	Microspore cytoplasm
Mi	Mitochondrion
MSP	Microspore surface pockets
N	Nucleus
P	Plastid
PM	Plasma membrane
PS	Periplasmic space
RER	Rough endoplasmic reticulum
RV	Reticulum vesicles
SER	Smooth endoplasmic reticulum
Ta	Tapetum
V	Vacuole

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Introduction

Representatives of the genus *Passiflora* have been extensively examined in many respects. For example, microsporogenesis, microgametogenesis and pollen morphology in *Passiflora* species were studied by García and coauthors (García et al. 2002) by light microscopy. Thanikaimoni (1986) analysed phylogenetic aspects of pollen apertures in *Passiflora*, among other genera. The molecular phylogeny of the genus *Passiflora* was discussed by Yockteng and Nadot (2004). Vegetative morphology, anatomy of flowers, pollen morphology and other characteristics of the family Passifloraceae were reviewed by Feuillet and MacDougal (2007). The palynotaxonomy of 21 Brazilian species of *Passiflora* L. was studied by Milward-de-Azevedo et al. (2010) using light microscopy and scanning electron microscopy, contributing to a better characterization of subgenus, species and subspecies. However, no ontogenetic studies of exine development using transmission electron microscopy have been undertaken until now. The pollen and aperture type (comprising three ring-like apertures or three large pores with opercula) in *Passiflora racemosa* have been described by Buchner and Halbritter (2000).

Many attempts have been undertaken to elucidate the mechanisms involved in the formation of the exine (one of the most complex biological patterns) in pollen and spores. These ontogenetic studies were initiated by Rowley and Flynn (1968) and Heslop-Harrison (1971, 1972), and extended by Dickinson and coauthors (e.g., Dickinson and Sheldon 1986) and by many others. There are several models of exine substructure, among them a poly-component rod-like model with a core and spiral binder unit (a tuft; Rowley et al. 1981; Rowley 1990), which was confirmed in experiments on oxidative degradation (Nowicke et al. 1986). Elongated units were postulated in the base of the exine and a viscin thread architecture by Hesse (1984). Blackmore and Claugher (1987) and Blackmore (1990) discovered two levels of exine substructure, the so-called “boundary layer”, and a finer substructure consisting of rod-like units. The rod-like model was confirmed in studies using atomic force and scanning tunnelling microscopy in a number of species, but in *Alnus* clusters of spheroids were detected (Rowley et al. 1995; Wittborn et al. 1996). Interconnected granules were revealed in exines in destructive experiments with 2-amino-ethanol (Southworth 1985a, b, 1986), threads and granules by a freeze-cleavage method (Takahashi 1993), and octahedron-like units (Kedves 1986; Kedves and Kedves 1999). Thus, the exine units mentioned most often are rods and granules. This cannot be a coincidence.

The important idea that self-assembling physicochemical interactions interfere into pattern formation in nature was put forward about a century ago (Thompson 1917) and

was then developed by many biologists (see reviews Blackmore et al. 2007). This idea was worked out in the field of sporoderm development (Gerasimova-Navashina 1973; van Uffelen 1991; Hemsley et al. 1992; Collinson et al. 1993; Gabarayeva 1993; Hemsley and Griffiths 2000) and received some confirmation in modelling experiments (Hemsley et al. 1996a, b, 1998, 2000, 2003; Griffiths and Hemsley 2001; Moore et al. 2009). Our recent hypothesis develops the self-assembling idea in that all the processes of exine development in the periplasmic space are based on unfolding the sequence of the micellar mesophase of surface-active substances (Gabarayeva and Hemsley 2006; Hemsley and Gabarayeva 2007). Our subsequent studies in a number of species (papers after 2007, cited in the references) and reconsideration of the previous ones have provided evidence for this hypothesis.

The aim of this study was to find out whether the sequence of sporoderm developmental events provides additional evidence for our hypothesis. This paper deals with the tetrad period of exine development.

Materials and methods

Flower buds of *P. racemosa* Brot. were collected in the greenhouse of the Komarov Botanical Institute, St. Petersburg, over 3 years in order to cover all the main developmental stages for study. Stamens were fixed in 3 % glutaraldehyde and 2.5 % sucrose in 0.1 M cacodylate buffer, with the addition of lanthanum nitrate (1 %) for better preservation of the plasma membrane glycocalyx, at pH 7.4 and 20 °C for 24 h. After post-fixation in 2 % osmium tetroxide (20 °C, 4 h) and acetone dehydration, the samples were embedded in a mixture of Epon and Araldite. Ultrathin sections were stained with a 2 % aqueous solution of uranyl acetate and 0.2 % lead citrate. Sections were examined with a Hitachi H-600 transmission electron microscope.

Results

Early tetrad stage

After completion of meiosis the early tetrad microspores have a very complicated surface with the appearance of finger-like invaginations and subsequent secondary invagination of portions of the microspore surface, leading to the formation of cytoplasmic pockets. Before all these changes of the microspore surface the whole microspore plasma membrane is encrusted with dark droplets, so that all the membranes of the changed, complicated microspore surface become covered by osmiophilic globules (Fig. 1a, d,

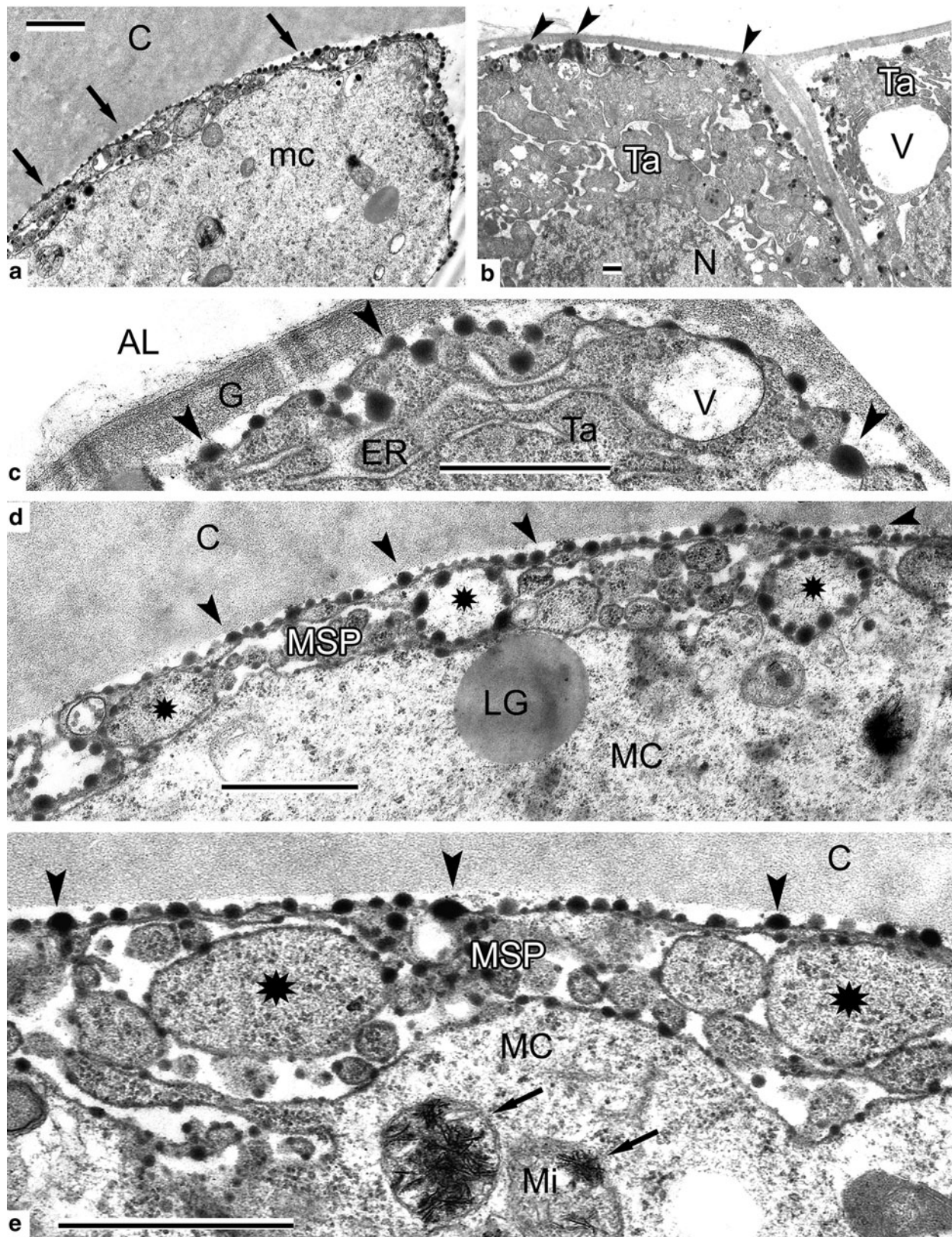


Fig. 1 Early tetrad stage. **a** A fragment of a microspore with dark droplets on the plasma membrane (*arrows*). **b, c** Tapetal cells with dark droplets (*arrowheads*) on the plasma membrane and in the peripheral cytoplasm. **d, e** The surface of a microspore of complex

configuration: groups of finger-like invaginations (*asterisks*) were subsequently secondarily invaginated to form pockets. Osmiophilic globules cover the plasma membrane. Needle-like crystals are seen in the mitochondria (*e arrows*). Scale bars 1 μ m

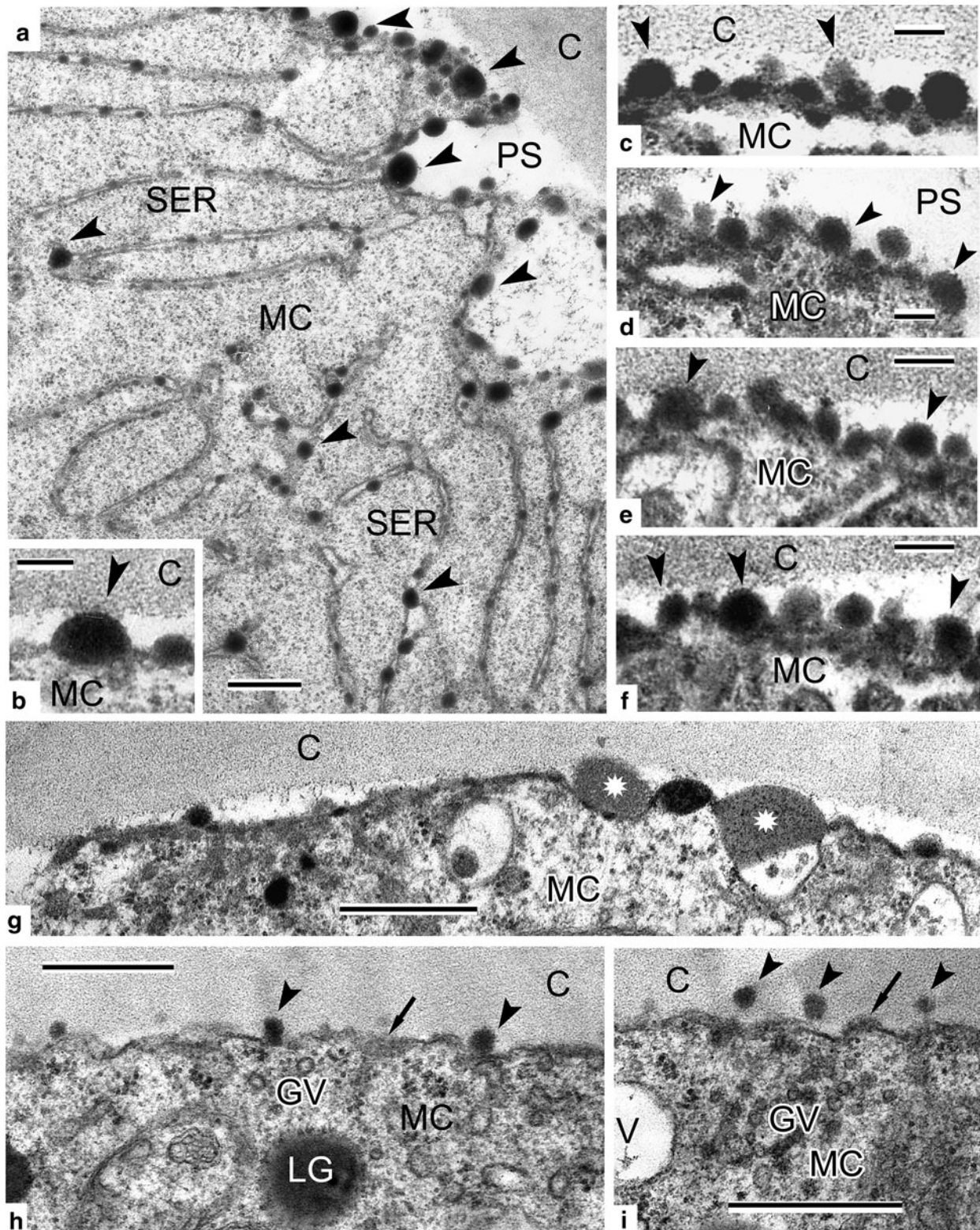


Fig. 2 Early tetrad stage. **a** The peripheral part of a microspore. Osmiophilic globules (*arrowheads*) are associated with cisternae of the smooth endoplasmic reticulum. **b–f** High magnification (about $\times 100,000$) reveals a cogged corona on the surface of the globules (*arrowheads*). **g** Coalescence of osmiophilic globules to form larger

ones (*asterisks*). **h, i** Gradual dissociation of the osmiophilic globules (*arrowheads*). Golgi vesicles appear under the plasma membrane and the first portions of the glycocalyx form (*arrows*). Scale bars **a, g–i** 0.5 μm ; **b–f** 0.1 μm

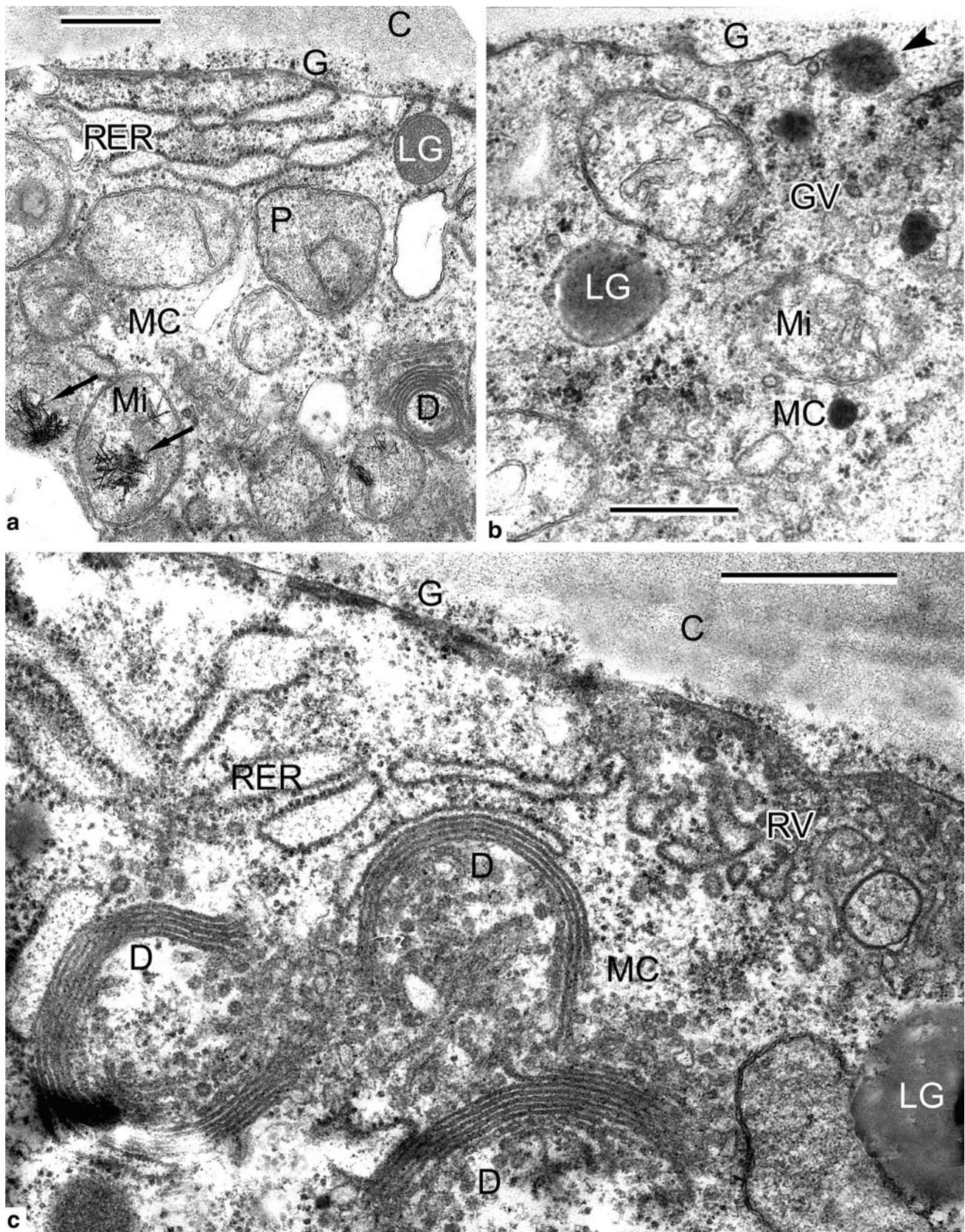


Fig. 3 Young tetrad stage. Appearance of the thin glycocalyx layer on the plasma membrane. Cisternae of the RER (a, c) and many active dictyosomes and their vesicles are observed in the peripheral cytoplasm. Crystals are seen in the mitochondria (a, arrows). Scale bars 0.5 μ m

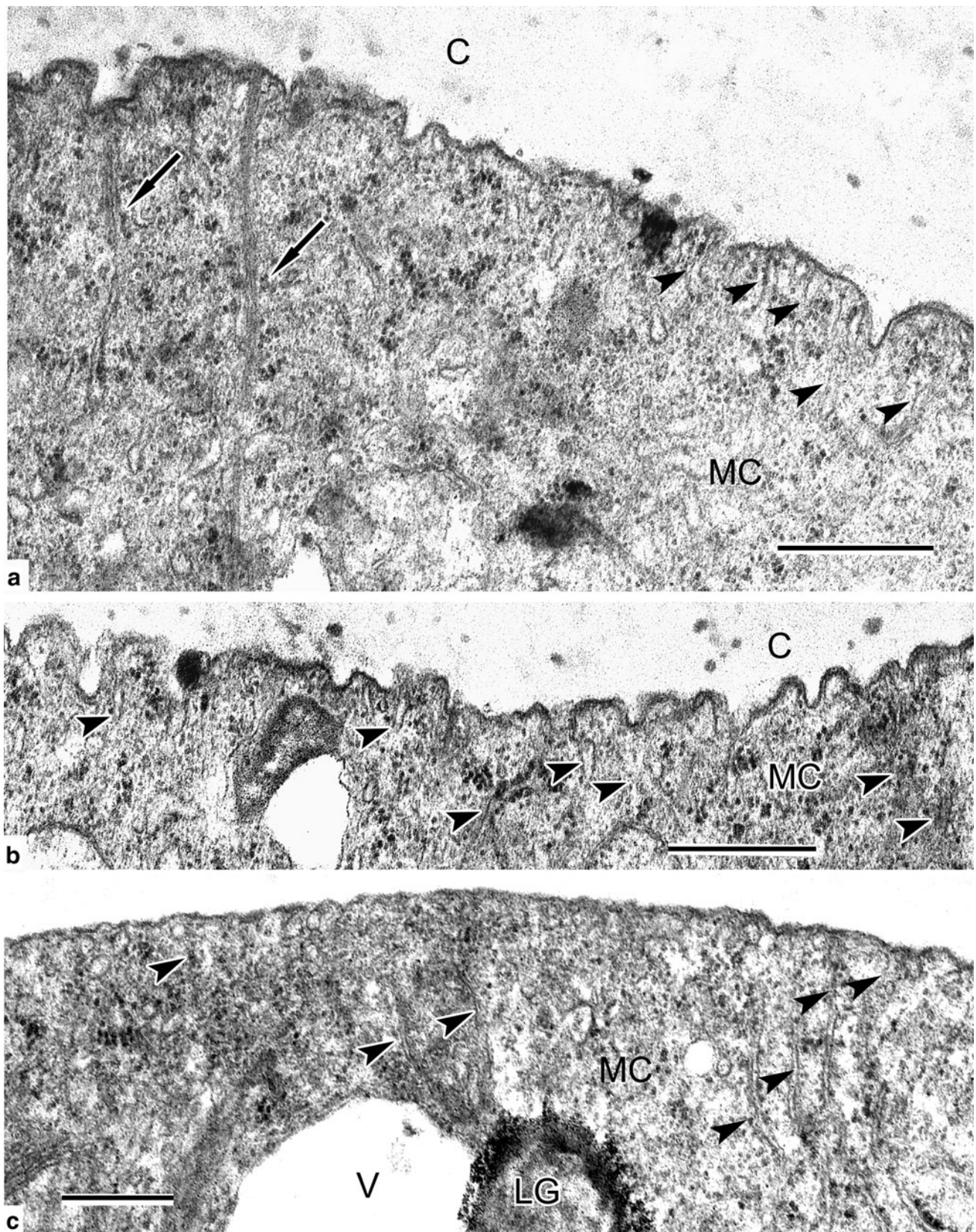


Fig. 4 Young tetrad stage. Bundles of microfilaments (**a**, *arrow*) and cisternae of the RER (*arrowheads*) are arranged perpendicular to the plasma membrane. *Scale bars* 0.5 μm

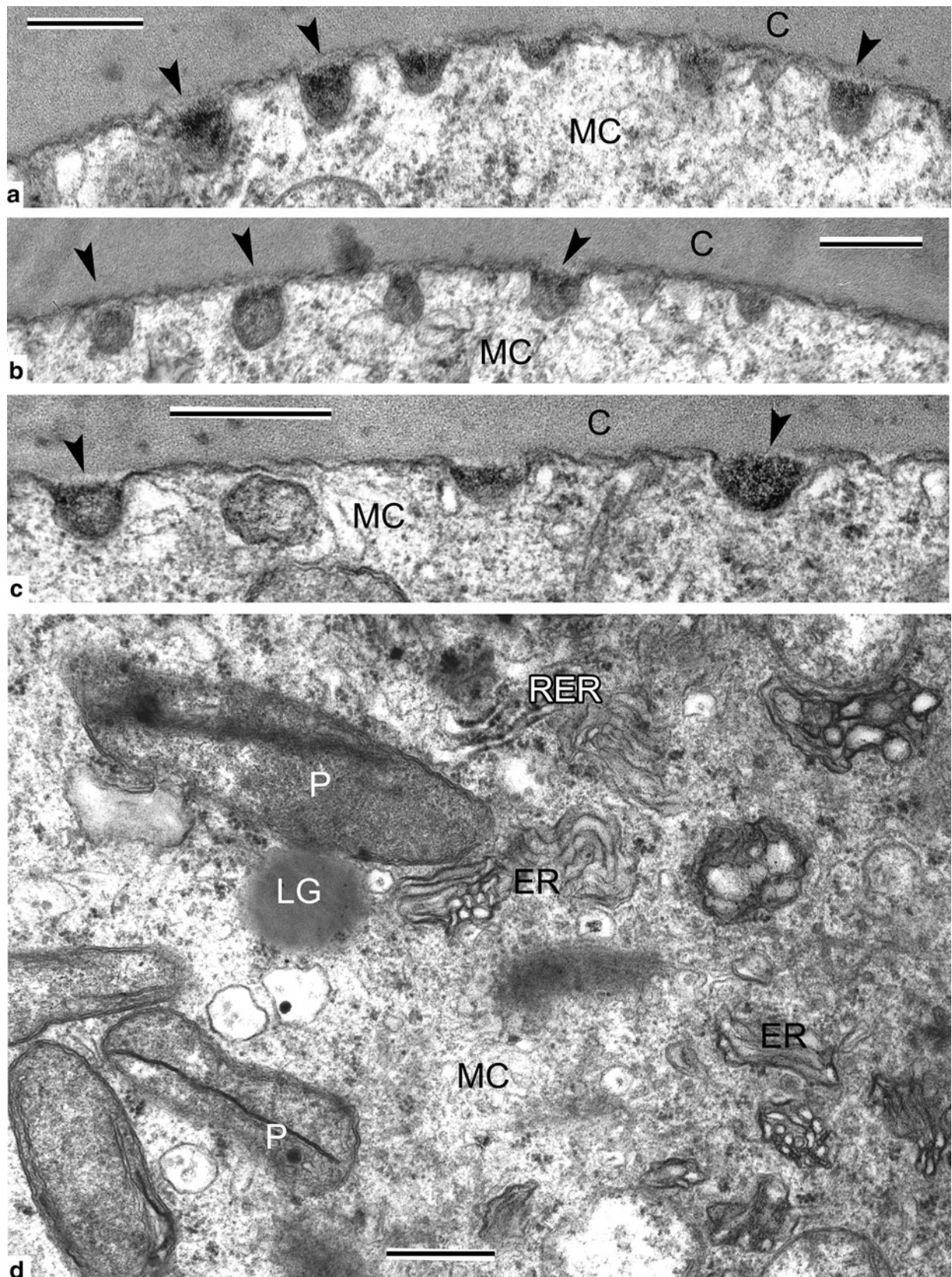


Fig. 5 Transition to middle tetrad stage. **a–c** Regularly disposed cup-like invaginations of the microspore surface are filled with the microgranular glycocalyx (*arrowheads*). **d** The microspore cytoplasm contains plastids, lipid globules and curved stacks of ER. Scale bars 0.5 μm

e). The locular side of the tapetal plasma membrane is also covered with dark droplets (Fig. 1b, c), and the latter are also observed in the peripheral cytoplasm (Fig. 1c). Needle-like crystals are observed inside the microspore mitochondria (Fig. 1e).

The peripheral microspore cytoplasm is pierced by cisternae of the smooth endoplasmic reticulum (SER), and many osmiophilic globules are associated with them (Fig. 2a). Higher magnification makes observation of these globules in detail possible: they have clogged coronae (Fig. 2b–f, arrowheads). Somewhat later some of these globules coalesce to form larger ones (Fig. 2g), but others gradually dissociate, decreasing in size. Many Golgi vesicles appear under the plasma membrane, and simultaneously first “islets” of the glycocalyx appear on the plasma membrane surface (Fig. 2h, i, arrows).

Young tetrad stage

A very thin glycocalyx layer appears on the plasma membrane (Fig. 3). The peripheral cytoplasm is full of the rough endoplasmic reticulum (RER) cisternae (Fig. 3a, c) and their vesicles (Fig. 3c), active dictyosomes (Fig. 3c) and their vesicles (Fig. 3b, c), and ribosomes and lipid globules.

Somewhat later the profile of the plasma membrane becomes wavy, and the system of microfilaments (MF) and RER cisternae is highly ordered: bundles of MF (Fig. 4a, arrows) and RER cisternae (Fig. 4a–c, arrowheads) are arranged radially, so that they are disposed perpendicular to the plasma membrane and in contact with it.

Middle tetrad stage

At the beginning of this stage regularly disposed cup-like invaginations of the plasma membrane appear, filled with microgranular contents (Fig. 5a–c, arrowheads). The latter are clusters of the glycocalyx. Many invaginations are in contact with RER cisternae. Specific curved stacks of RER cisternae crowd the microspore cytoplasm (Fig. 5d). There are also many lipid globules, plastids, dictyosomes and ribosomes in the cytoplasm.

Somewhat later the diameter of cup-like invaginations with the microgranular glycocalyx, shown in Fig. 5a–c, increases, and clusters of the glycocalyx acquire a lens-like form (Fig. 6a, arrowheads). The microspore cytoplasm is full of plastids, dictyosomes, RER cisternae, lipid globules, mitochondria and ribosomes. The glycocalyx structure changes gradually from microgranular to microfibrillar (Fig. 6b, c). Bundles of microtubules (MT; Fig. 6b, arrowhead) and MF (Fig. 6b, arrows) are oriented perpendicular to the plasma membrane. The latter lack glycocalyx between the glycocalyx clusters (Fig. 6b, c).

Fig. 6 Middle tetrad stage. **a** Fragments of two microspores. Cup-like invaginations increase in diameter and acquire a lens-like form (arrowheads). **b, c** The substructure of the glycocalyx inside the invaginations changes gradually from microgranular to fibrillar. Between the glycocalyx “lens” the plasma membrane lacks glycocalyx; bundles of microtubules (Fig. 6b, arrowhead) and microfilaments (**b**, arrows) and cisternae of the RER (**c**, arrows) are disposed perpendicular to the plasmalemma. Scale bars 0.5 μm

Instead, cisternae of the RER are disposed at these sites (Fig. 6c, arrows).

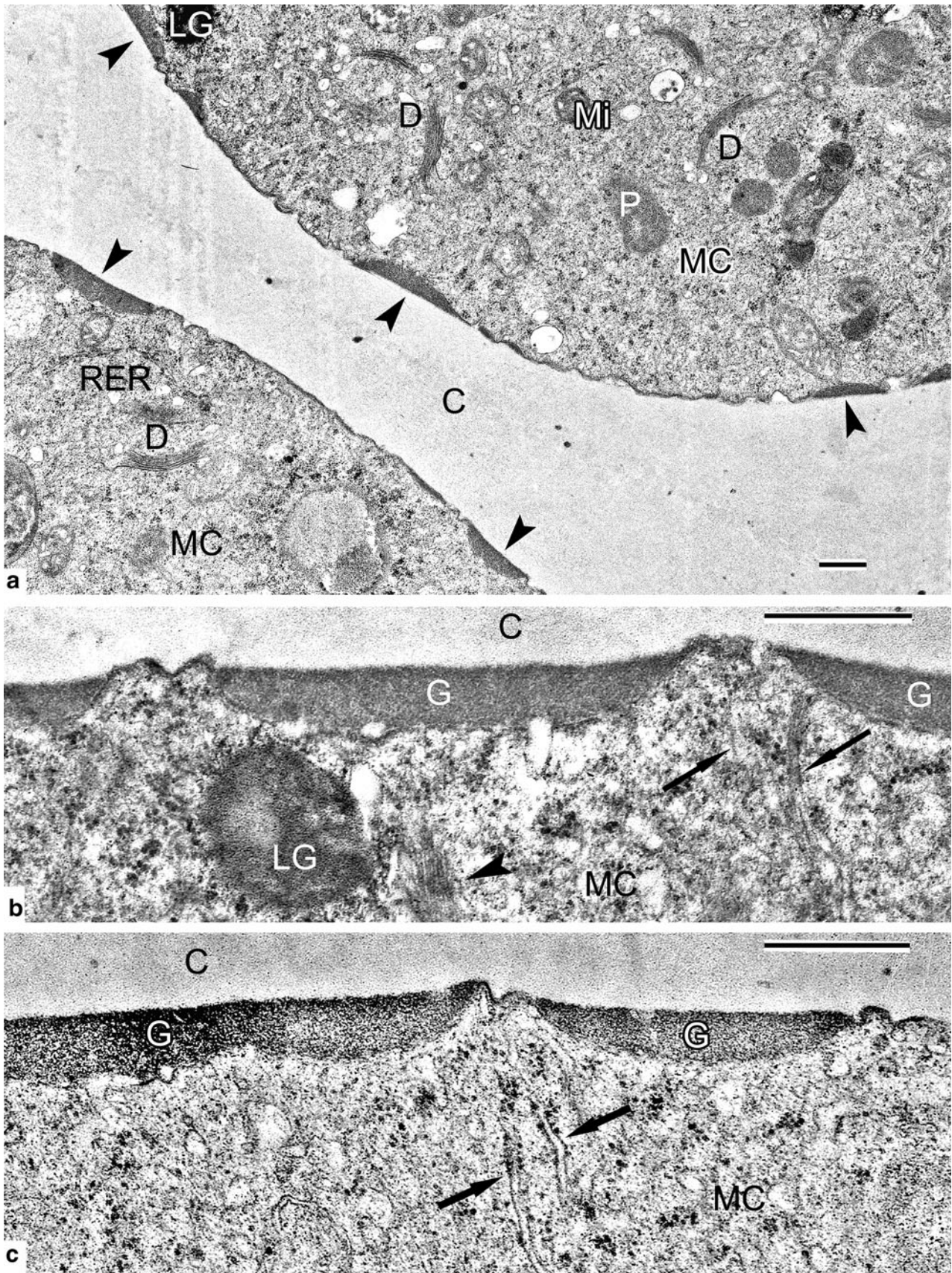
At the next ontogenetic moment the substructure of the glycocalyx clusters becomes apparent: many tubule-like units are formed, most of them radially oriented (Fig. 7a–c, arrowheads). Many cytoplasmic organelles and polysomes fill up the microspore the cytoplasm (Fig. 7b). The tapetum has a well-developed glycocalyx and extensively developed RER (Fig. 7d).

Late tetrad stage

At the transition to the late tetrad stage the second layer of the glycocalyx appears on the plasma membrane as the site of formation of the procolumellae (Fig. 8). This inner newly formed layer, the glycocalyx-2 layer, spreads along the whole microspore surface, with the exception of the sites of future apertures (Fig. 8b, asterisk). The glycocalyx-2 layer is denser, and shows greater contrast than the glycocalyx-1 layer (Fig. 8a–f). The substructure of this second layer of the glycocalyx is the same as that of the first layer: radially oriented tubule-like units (Fig. 8e, f, white arrowheads). Note the electron-transparent axial gap inside the procolumellae (Fig. 8d, e).

Somewhat later the definite portions of the microspore surface invaginate rather deeply (Fig. 9a, arrows). These are sites of the future ring-like apertures (the section shown in Fig. 9a has gone through one of three future apertures). Lens-like clusters of the glycocalyx-1 layer are readily discernible (Fig. 9a, arrowheads). The microspore cytoplasm is crowded with organelles, and groups of undulating stacked RER cisternae are of particular note (Fig. 9a, b).

The details of the glycocalyx substructure are shown in Fig. 10. The glycocalyx-1 layer which consists at first of radially oriented units (Fig. 10a) becomes gradually less compact, whereas the glycocalyx-2 layer remains dense (Fig. 10b–d). Note the electron-transparent gaps inside tectate procolumellae (future constituents of the muri; Fig. 10b, c, arrowheads). At higher magnifications the details of the glycocalyx-1 substructure become apparent: radially oriented units look like spirals (some of them indicated by arrowheads in Fig. 11a and at higher magnification in Fig. 11b). At this ontogenetic moment the central parts of the glycocalyx-1 clusters become friable and lose their contrast (Fig. 11c, asterisk; see also Fig. 11a, e).



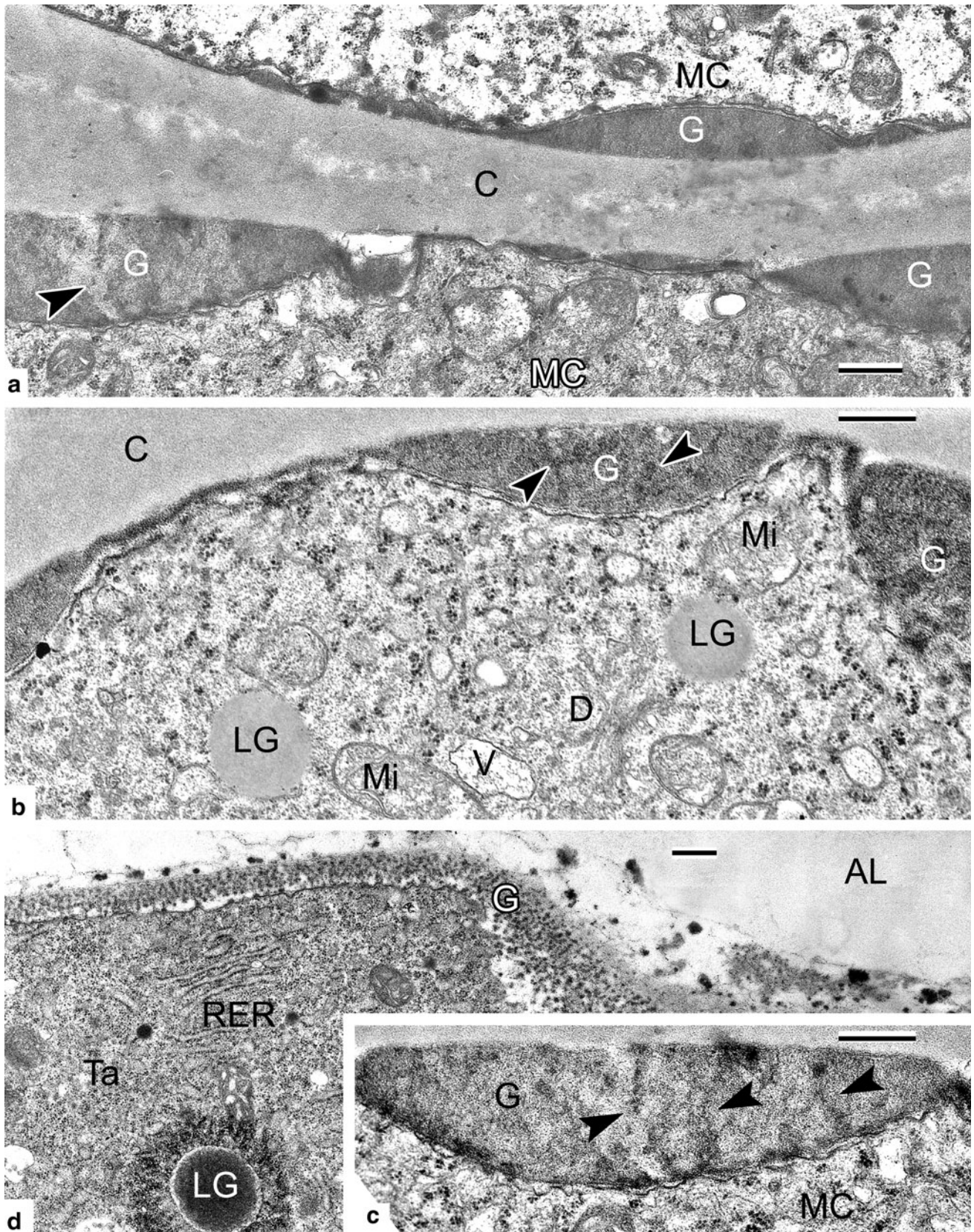


Fig. 7 Middle tetrad stage in progress. **a–c** Substructure of the glycocalyx become apparent. It consists of many radially oriented tubule-like units (arrowheads). **b** The cytoplasm is rich in organelles and polysomes. **d** A tapetal cell with its glycocalyx and extensively developed RER. Scale bars 0.5 μm

Plastids with dark globules between the two restricting membranes are observed in the microspore cytoplasm (Fig. 11d).

At the end of the tetrad period the invaginated portions of the microspore surface (future aperture sites) are especially prominent (Fig. 12a). These sites lack the glycocalyx-1 layer, and the glycocalyx-2 layer is less prominent here (Fig. 12a, asterisks). The inner surfaces of the tapetal cells bear the glycocalyx, and the cytoplasm is packed with ribosomes, active dictyosomes, RER cisternae and plastids, and contains vacuoles (Fig. 12b).

Discussion

Prepattern stages

As shown above, the first elements, appearing on the microspore plasma membrane during the early tetrad stage, are dark globules of some lipid substance encrusting the highly uneven microspore surface (Fig. 1a, d, e). A complex configuration of the microspore surface, resulting from the local finger-like invaginations of the microspore surface followed by invagination of larger portions of the surface and leading to the appearance of the cytoplasmic pockets, is characteristic of cells with active surface processes such as uptake. The parietal secretory tapetal cells also bear globules of lipid substances on their plasma membrane (Fig. 1b, c), and it is highly probable that this substance has been secreted by the tapetum, excreted into the anther loculus and taken up by the microspores. Similar globules have been observed at this initial tetrad stage in many species (Dickinson 1976a, b; Hesse 1985; El-Ghazaly and Jensen 1987; Skvarla and Rowley 1987; Gabarayeva 1991, 1995, 2000; Rowley et al. 1992; Takahashi 1993; Gabarayeva and Rowley 1994; Gabarayeva and El-Ghazaly 1997; Gabarayeva and Grigorjeva 2002, 2003, 2010; Gabarayeva et al. 2009), and their appearance can be regarded as a regular feature of the early tetrad period.

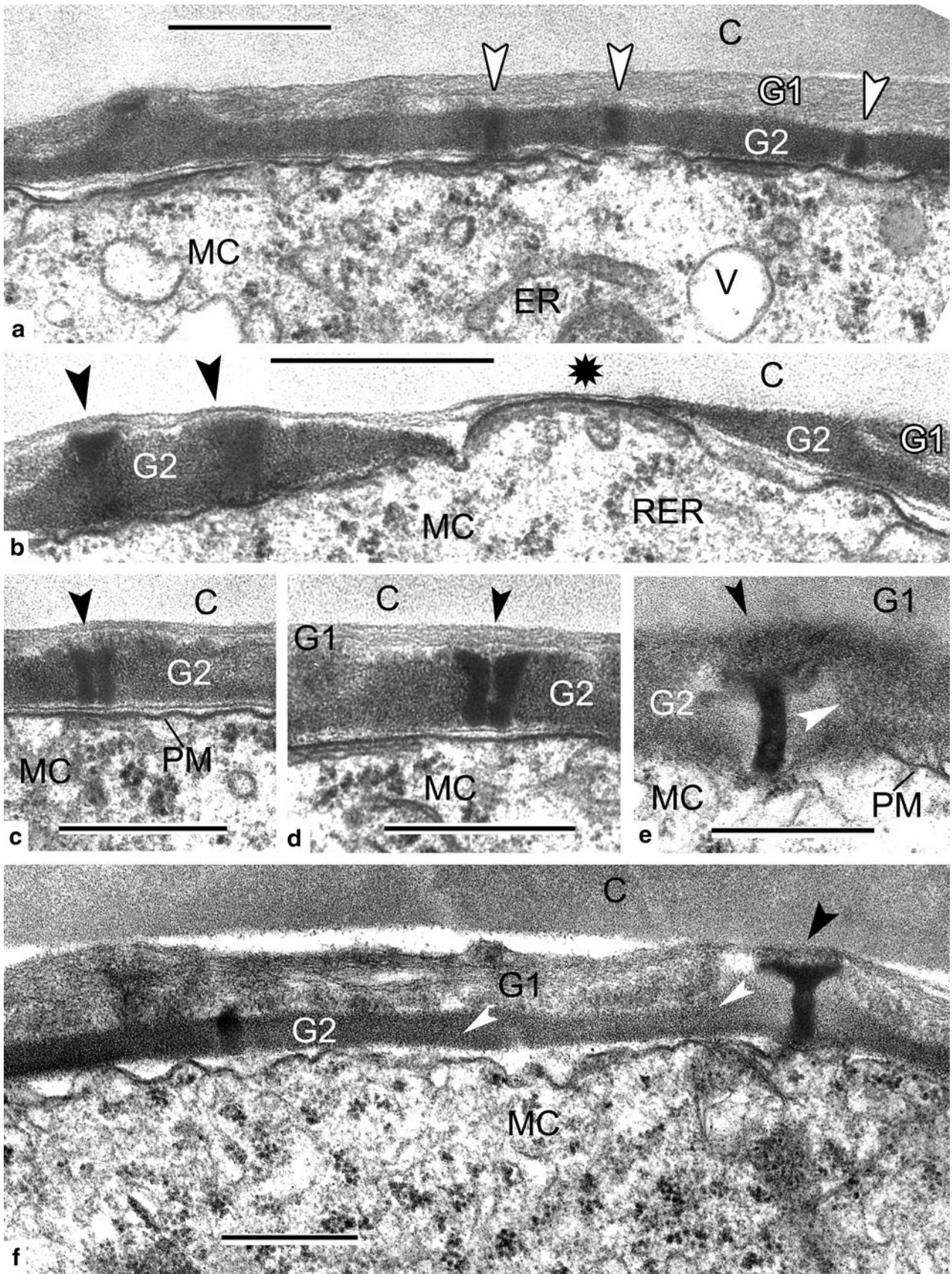
However, a peculiar feature is that not only tapetal cells, but also microspores themselves synthesize lipid globules. They are observed inside the cisternae of SER, localized in the peripheral cytoplasm, from where they are excreted on the microspore surface (Fig. 2a, arrowheads). High magnification (about $\times 100,000$) shows the substructural details of these globules: they have a cogged border (Fig. 2b–f) and consist of a dark core and a lighter halo (Fig. 2f). The same substructure of the osmiophilic globules has been observed in many other species, including species studied by us: *Stangeria eriopus* (Gabarayeva and Grigorjeva 2002), *Illicium floridanum* (Gabarayeva and Grigorjeva 2003), *Trevesia burckii* (Gabarayeva et al. 2009), *Chamaedorea microspadix* (Gabarayeva and Grigorjeva 2010),

and *Persea americana* (Gabarayeva et al. 2010) at the same ontogenetic stage. These globules may contain either lipophilic nutritive substance of tapetal origin or lipoproteins originating from the microspore SER cisternae (Fig. 2b). These globules are temporary structures, and they are either absorbed during the process of uptake or dissociate in the periplasmic space between the callose jacket and the plasma membrane for further processing.

Initiation of the exine pattern

Golgi vesicles add an important ingredient to the medium of the periplasmic space, delivering the main glycocalyx components, that is glycoproteins (Rowley 1971; Pettitt and Jermy 1974; Rowley and Dahl 1977; Pettitt 1979). Most of the glycoproteins are surfactants. Lipopolysaccharides (which are also mainly surfactants) have also been detected in the periplasmic space as constituents of the glycocalyx (Rowley 1975). The initial glycocalyx looks like a disordered fibrillar layer (Fig. 3), but this stage is a key one for subsequent development of the pollen wall. The position of the future luminae and muri of the exine reticulum are predestined by the cytoskeleton: an ordered picture of the arrangement of MF bundles is evident (Fig. 4). The RER cisternae are also arranged uniformly: both MF and MT bundles and RER cisternae are disposed perpendicular to the plasma membrane, creating radial rays in the microspore cell. Such disposition of cytoskeletal elements (see also Fig. 6b) causes the appearance of regularly disposed invaginations of the plasma membrane, first small (cup-like; Fig. 5a–c), then larger ones (lens-like; Fig. 6). In species with a reticulate exine, the initial undulating conformation of the plasma membrane plays an important role in pattern establishment; such species include, for example, *Illicium* and *Schizandra* (Gabarayeva and Grigorjeva 2003) and *Trevesia* (Gabarayeva et al. 2009).

Invaginations of the plasmalemma appear as a consequence of pulling inside some portions of the plasma membrane by contraction of MF, anchored by their other end in the nuclear envelope, with the simultaneous resistance of MT. The radial system of MT extending between the nuclear envelope and the plasma membrane during early male haplophase was described by Dickinson and Sheldon (1984). Spatial and temporal correlations have been found between the distributional pattern of cortical MT and coarsely reticulate exine patterns in *Vigna vexillata* (Muñoz et al. 1995). Working on microspore development in *Vigna unguiculata*, Southworth and Jernstedt (1995) concluded that MT do not determine exine pattern—on the contrary, the developing pattern precedes rearrangement of MT. The authors believe that the pattern formed is a response to the tensile and rigid properties of



◀ **Fig. 8** Late tetrad stage. Appearance of the second layer of the glycocalyx. The newly formed inner layer, the glycocalyx-2 layer, spreads along the whole microspore surface except for the sites of future apertures (**b**, *asterisk*). It is denser than the outer glycocalyx, but has the same substructure: radially oriented tubule-like units (**e**, **f**, *white arrowheads*). In the intervals between the lens-like glycocalyx-1 layer, the glycocalyx-2 layer is thicker (**e**, **f**). Initial procolumellae appear (*arrowheads*), first as “shadows” (**a**, **b**), then more and more distinct (**e–f**, *black arrowheads*). These procolumellae differ from each other: those disposed under the “lens” of the glycocalyx-1 layer are small and atectate (**a–d**), and those between the “lenses” are higher and covered with tectum (**e**, **f**, *arrowhead*). Scale bars 0.5 μm

the cytoskeleton (tensegrity) and to osmotic pressure in the microspore, balanced against the pressure and volume of newly secreted matrix. This model of pollen wall patterning includes a self-patterning idea and suggests that semi-stiff MT bend or move to areas of plasma membrane protrusions, allowing greater indentation of the plasma membrane in other regions (Southworth and Jernstedt 1995).

Plant cell wall (especially the microspore glycocalyx, or primexine matrix) can be considered homologous with animal cell extracellular matrix, and the idea of Wyatt and Carpita (1993) that the cytoskeleton–cell-wall continuum is a real system involved in the appearance of periodic plasma membrane invaginations, can also be applied to microspores (The term “extracellular matrix” should be replaced with the term “exocellular matrix”, because the cell surface coating, the glycocalyx, is an integral part of a cell.) Current knowledge points to interaction of the cytoskeleton and ECM via transmembrane proteins of the plasma membrane. An important feature of the cytoskeleton–ECM continuum is adhesion, a physical attachment of the plasma membrane to the wall (in our case, to the glycocalyx). It has been shown that the ECM receptor, integrin β_1 , induces the formation focal adhesions and supports a force-dependent stiffening response, whereas non-adhesion receptors do not (Wang et al. 1993). Large-scale changes in cell and nuclear shape result from the action of mechanical tension that is generated within the cytoskeleton via an actomyosin filament sliding mechanism and transmitted across integrin receptors of the plasma membrane. It is physically resisted by immobilized adhesion sites within the ECM/exocellular matrix (Sims et al. 1992).

Tensegrity models mimic this response, suggesting that integrins act as mechanoreceptors and transmit mechanical signals to the cytoskeleton (Ingber and Jamieson 1985; Sims et al. 1992; Ingber 1993). The term “tensegrity” (tensional integrity) came from architecture (for more detailed information on tensegrity, see Gabarayeva et al. 2009). Recent work has provided strong evidence to support the use of tensegrity by cells (see reviews by Ingber 2003a, b). The cellular tensegrity model proposes that the whole cell is a prestressed tensegrity structure. In other

words, cells generate their own internal tension or prestress in the actin cytoskeleton (MF pull the plasma membrane inside), which is balanced by internal MT struts (MT do not allow the plasma membrane to sag inside too much) and external ECM adhesions. These focal adhesions represent discrete points of cytoskeletal insertion on the ECM analogous to muscle insertion sites on bones (Ingber 2003a).

In *Passiflora*, clusters of the first generation of the glycocalyx (glycocalyx-1) accumulate inside invaginations (as in *Illicium*, *Schizandra* and *Trevesia*) initially in the form of osmiophilic microgranules (Fig. 5a–c) and are later rearranged into lens-like clusters with radially oriented rod-like units (Figs. 6, 7). These clusters of the glycocalyx-1 act to support the wavy profile of the future reticulate exine pattern, more precisely—deepenings of the network. The glycocalyx-1 is not a framework for future columellae and muri and disappears later in development. Only species with reticulate patterns have in their ontogeny a period (about the middle/late tetrad stages), characterized by the glycocalyx, of arrested growth in invaginations and promoted growth on pinnacles. The reason for this arrest is the loss of contact between the glycocalyx and plasma membrane. The glycocalyx is an integral part of the plasmalemma and cannot exist in a functional condition, being isolated from it. In *Passiflora*, the clusters of glycocalyx-1 become separated from the plasma membrane by the glycocalyx-2 layer, and the consequence of this is first the loosening of its structure (Fig. 11a), and then its disintegration in the post-tetrad period. The same happens with the glycocalyx in *Schisandra* (Figs. 27, 28 in Gabarayeva and Grigorjeva 2003) and in *Trevesia* (Plate V, 2, 3 in Gabarayeva et al. 2009).

At a later stage osmiophilic granules inside invaginations gradually rearrange into rod-like units (Figs. 6b, c, 7b, c, 13)—described as tufts (Rowley and Dunbar 1970). An interesting point is that RER cisternae (which are arranged perpendicular to the plasma membrane and in contact with it between the clusters of the glycocalyx) probably prevent the delivery of the glycocalyx material from Golgi vesicles, and at these sites the plasmalemma lacks a glycocalyx.

The development of primexine

The glycocalyx-2 layer, which appears under the first glycocalyx layer, is responsible for all elements of the future exine. The first elements of the primexine are formed in the second layer of the glycocalyx which appears on the plasma membrane. This second glycocalyx layer, unlike the first one, is continuous along the whole microspore surface, exception for aperture sites (Fig. 8a, b). It is denser than, but has the same substructure as, the first layer, with radially oriented rod-like units (Fig. 8e, f). Initial

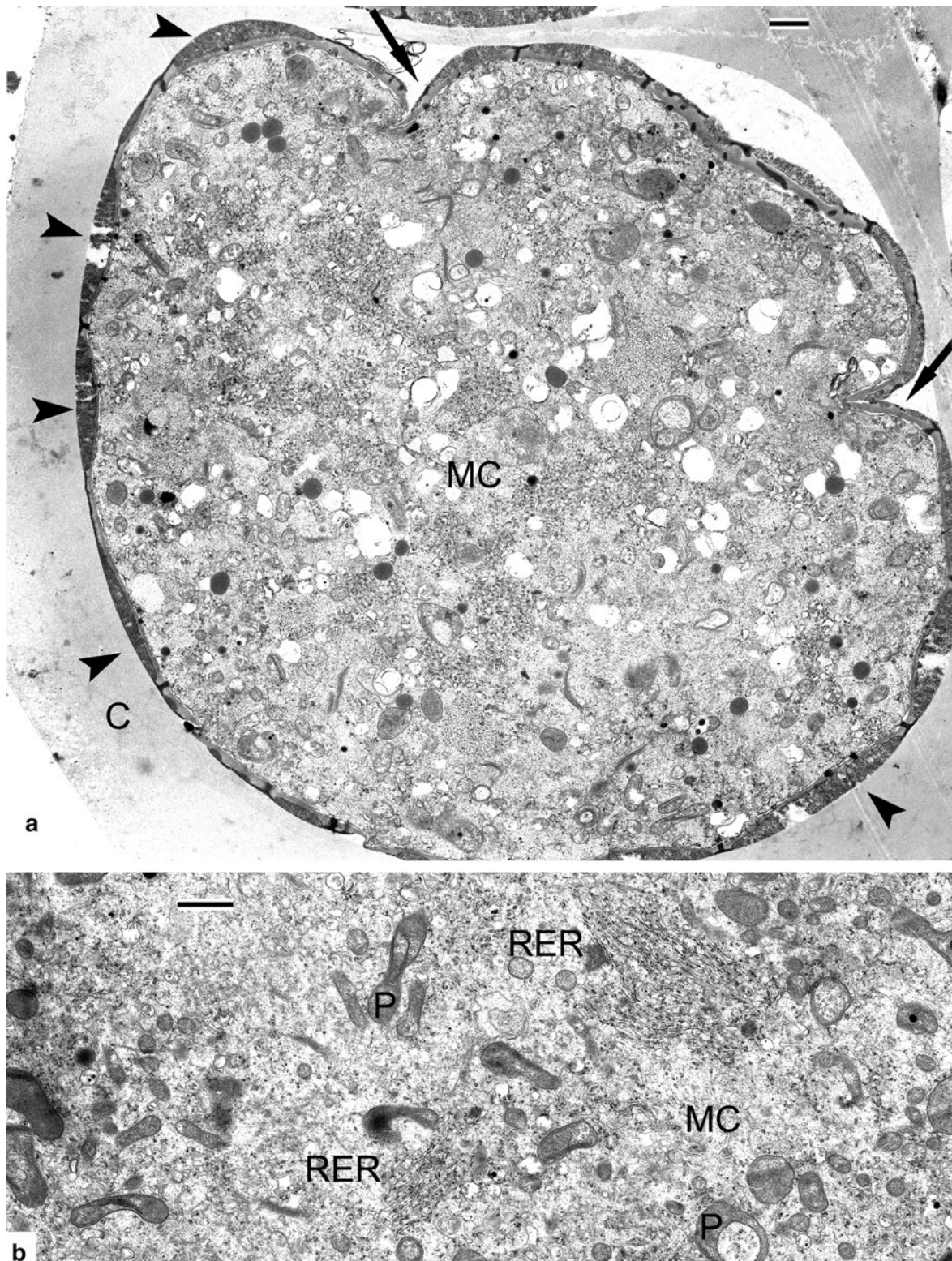


Fig. 9 Late tetrad stage in progress. **a** Overview of a microspore. Portions of the cell surface, predestined to be a circular apertures, are invaginated (*arrows*). Lens-like glyocalyx clusters are indicated by *arrowheads*. Many groups of stacked RER are seen in the cytoplasm.

b Portion of the cytoplasm at higher magnification. Many plastids, some of them dividing, and stacks of RER cisternae are discernible. *Scale bars* 1 μ m

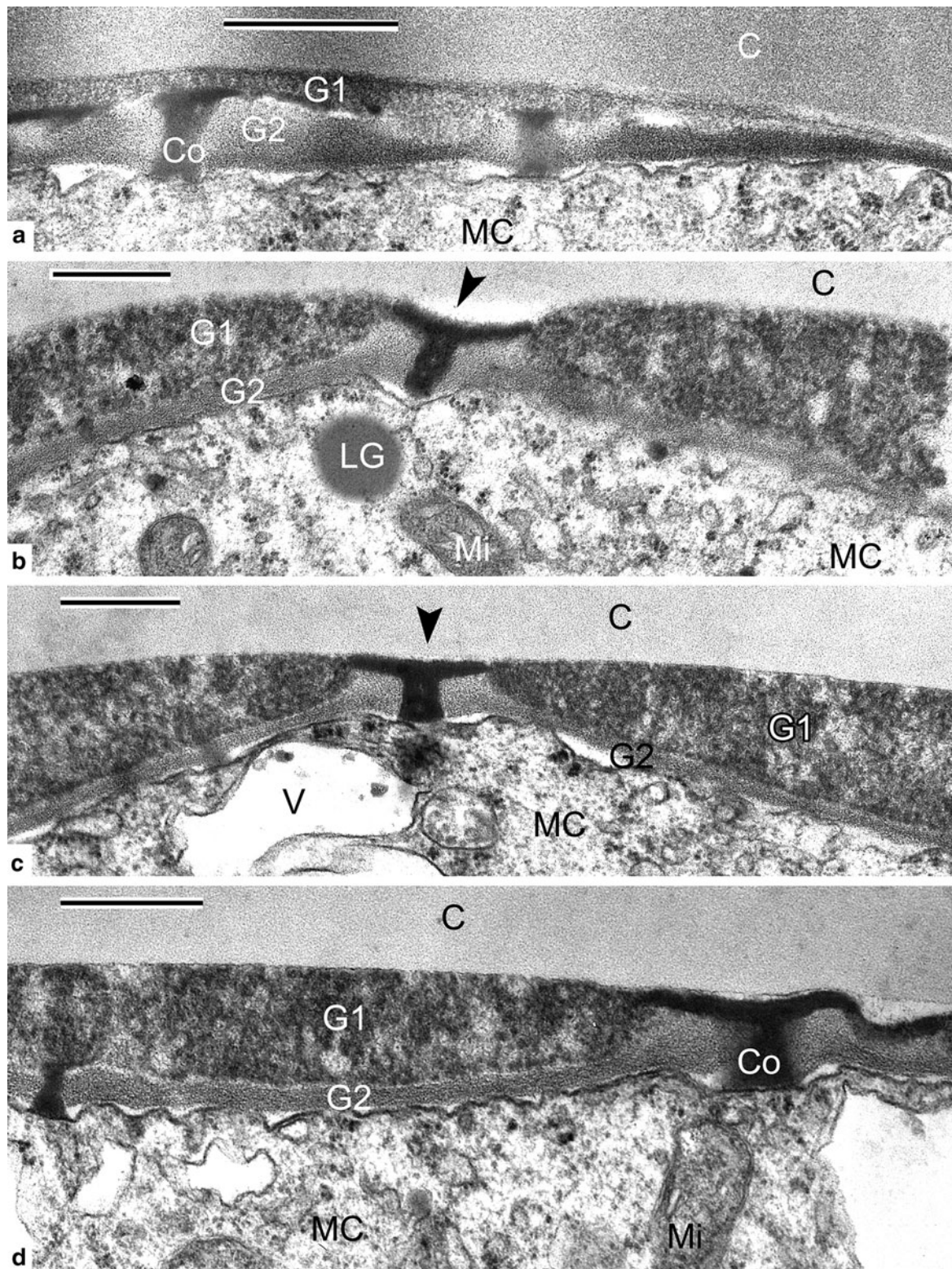


Fig. 10 Late tetrad stage (somewhat later than shown in Figs. 8 and 9). **a** The substructure of the first (outer) glycoalyx layer is still ordered and consists of tightly packed radial units. Atectate procolumellae are predestined to be small atectate columellae on the bottom of the lumina of the future reticulate exine pattern. **b–d** The

substructure of the glycoalyx-1 layer becomes loosely arranged. Tectate procolumellae between the lens-like clusters of the glycoalyx-1 layer form future muri of the exine reticulum (arrowheads). Scale bars 0.5 μm

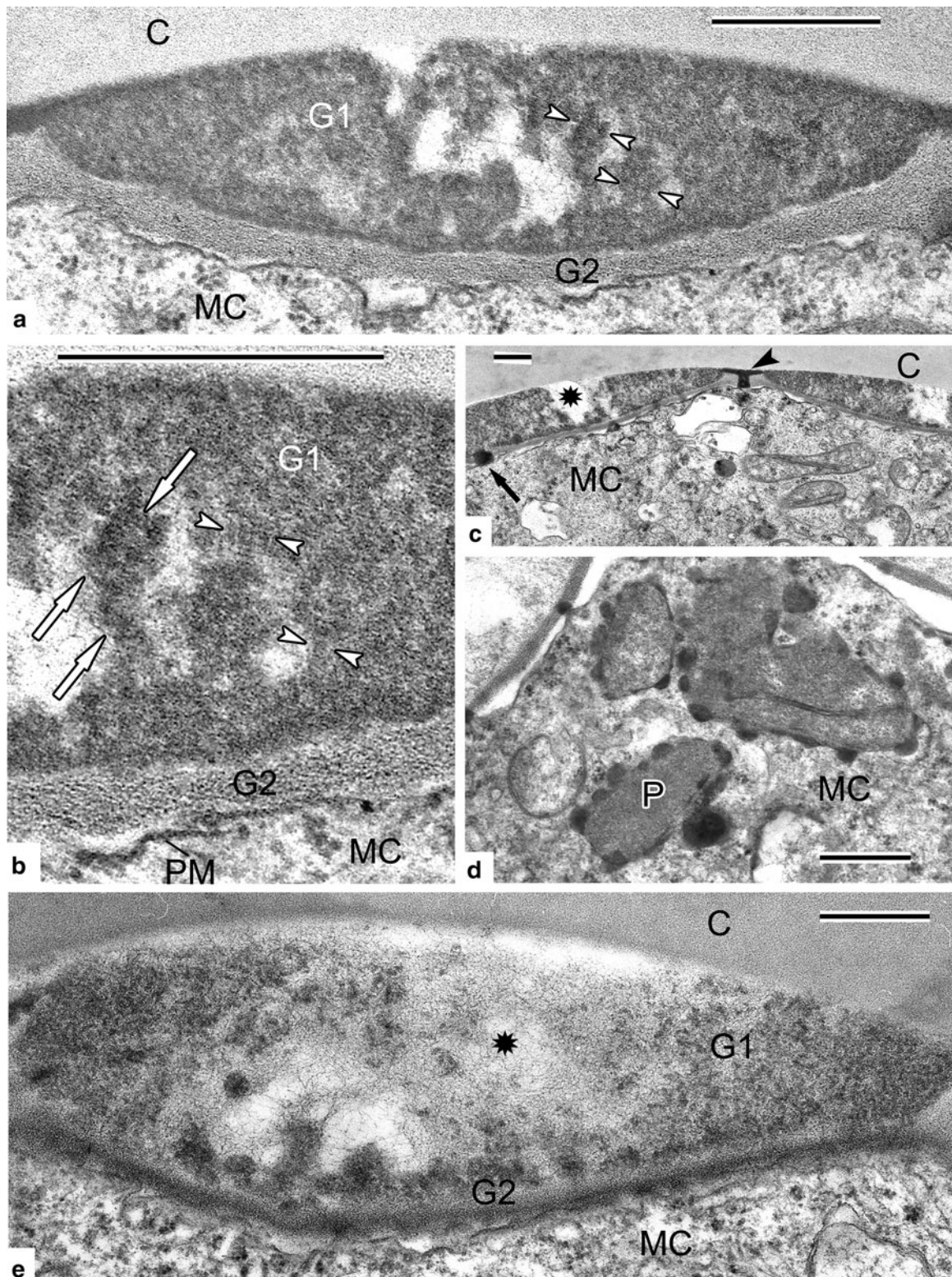


Fig. 11 End of the late tetrad stage. **a** Lens-like cluster of the glycoalkal-1 layer. The glycoalkal units are arranged more loosely, and their substructure is especially evident: tubule-like units show a spiral pattern (their extent indicated by *arrowheads* at opposite sides). **b** Magnified portion of **a** shows more clearly the spiral-like pattern of the radial units (*arrows* and *arrowheads*). **c**, **e** The border of

microspores. Lens-like glycoalkal-1 clusters have lost their compact, ordered structure, the central friable portions are weakly osmiophilic (**c**, **e**, *asterisks*). A tectate columella of a future murus is indicated by the *arrowhead* (**c**), and an atectate columella under a lens-like cluster by the *arrow*. **d** Plastids with dark droplets between the restricting membranes. Scale bars 0.5 μm

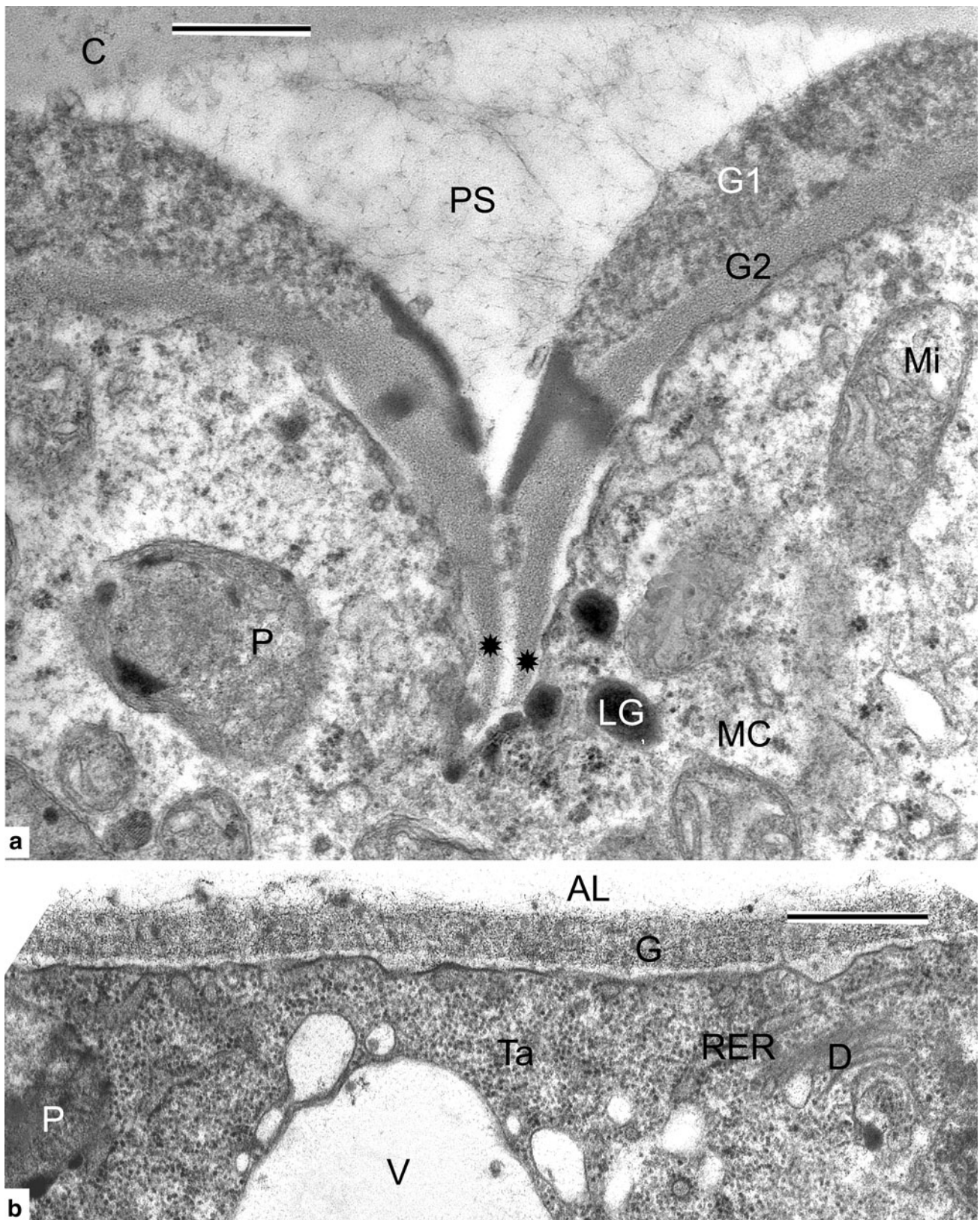


Fig. 12 Late tetrad stage. **a** An aperture site is deeply invaginated. The glycocalyx-2 layer is much thinner (*asterisks*), and the glycocalyx-1 layer is completely absent at this site. **b** A portion of a tapetal

cell. The tapetal glycocalyx is well developed, and the cytoplasm is full of ribosomes, RER cisternae, dictyosomes and plastids with osmiophilic droplets along the border. *Scale bars* 0.5 μ m

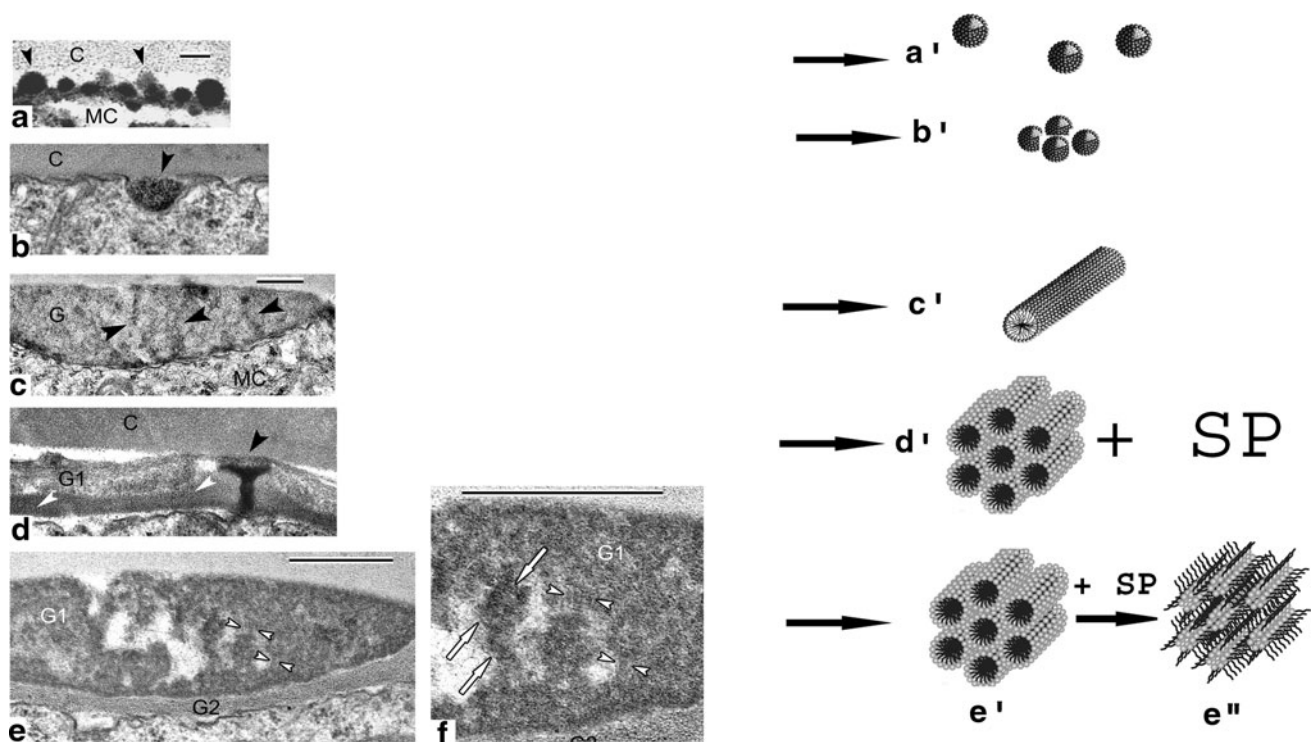


Fig. 13 Semischematic illustration showing the main developmental steps in the tetrad period of *P. racemosa* (left original micrographs reduced in size, right) micellar interpretation of the processes). *a* Early tetrad stage. Osmiophilic globules encrusting the microspore surface (see also Fig. 2b–f) correspond to spherical micelles (*a'*). *b* Young tetrad stage in progress. Microgranules inside cup-like invaginations (arrowhead; see also Fig. 5a–c) correspond to generation of small spherical micelles (*b'*). *c* Middle tetrad stage. Elongated units of the glycocalyx-1 (arrowheads; see also Fig. 7c) correspond to spherical micelles that have been rearranged to cylindrical micelles (*c'*). *d* Late tetrad stage. The appearance of the second, more dense

glycocalyx layer and formation of procolumellae in it (arrowheads; see also Fig. 8) corresponds to the next micellar mesophase—a layer of hexagonally packed cylindrical micelles, middle mesophase (*d'*). After sporopollenin (SP) accumulation on groups of micelles through this layer procolumellae appear. *e* The end of the tetrad period. The glycocalyx-1 units show readily discernible spiral substructure with a light core and a dark binder element. As the concentration of hydrophobic sporopollenin monomers in the periplasmic space increases, normal micelles turn inside out, opening a core hydrophilic channel (*e'*, *e''*). This channel is also seen as an axial gap inside procolumellae (Fig. 8c–e)

procolumellae appear first as shadows (Fig. 8a, b). Then as sporopollenin (SP) progressively accumulates, procolumellae become more prominent (Fig. 8c–e). These procolumellae, which are disposed under the lens-like glycocalyx clusters, are predestined to be atectate columellae on the “bottom” of luminae of the future reticulate exine (Fig. 8a–d, arrowheads). Higher tectate procolumellae, disposed between lens-like glycocalyx clusters, are the constituents of the future muri (Fig. 8e, f, arrowheads). An important point is that procolumellae are formed on the base of clusters of radially oriented glycocalyx rod-like units. This can be seen especially in the image of a procolumella in Fig. 8b (right arrowhead) and of the procolumella in Fig. 8e (black arrowhead): at the upper part of these procolumellae their subunits fan out. It is clear from these images that procolumellae include many tufts, so they are composite procolumellae. The central part of the procolumellae lack SP, and they look “hollow”. Similar composite procolumellae have been observed in *Schisandra*

(Gabarayeva and Grigorjeva 2003). “Hollow” composite procolumellae are also typical of the late tetrad stage in *Trevesia* (Gabarayeva et al. 2009, Plate VII, 3 and inset). Images like those in *Trevesia*, in which a large spiral twists around the procolumella, may demonstrate that several tufts packed together undergo redistribution of their subunits. Their cores become united, and their binder units form one common spiral around the whole procolumella. The same large spirals around columellae have been observed in *Cabomba* exine (Gabarayeva et al. 2003a). In *Passiflora*, such spirals are weakly outlined (black arrowheads in Figs. 8e and 10b, c), which is why the inner “hollow” axial parts look interrupted. It should be stressed that such rearrangement occurs not only with tufts of the composite procolumellae, but also through the whole glycocalyx: large outer spirals (arrows) and smaller inner spirals (arrowheads) are distinct in the first (outer) layer of the glycocalyx (Fig. 11a, b). This new arrangement of the glycocalyx units corresponds to the appearance of some super-tufts.

It is most probable that the outer “shells” of the primexine “hollow” procolumellae correspond to the receptive sites for receptor-dependent SP that first define the form of ectexinous structural elements, that is the “boundary layer” of Blackmore and Barnes (1987), whereas the inner parts correspond to receptor-independent SP (for these terms see Rowley and Claugher 1991). In destruction experiments using oxidation of mature exines with potassium permanganate after acetolysis we have confirmed this suggestion by showing the appearance of hollow columellae in *Lavatera* pollen walls and hollow alveolae in *Stangeria* alveolate exines, erosion increasing with the duration of treatment (Gabarayeva et al. 2003b).

Figure 8f (right white arrowhead) shows that one side of the radially oriented glycocalyx units is rooted into the cytoplasm and the other side into callose. This has been observed in an even more pronounced manner during the middle and late tetrad stages in several species, including *Trevesia* (Gabarayeva et al. 2009), *Magnolia sieboldii* (Gabarayeva and Grigorjeva 2012) and (especially prominent) *Symphytum* (Gabarayeva et al. 2011). This means that the role of callose as an organizer of the glycocalyx structure is essential. Callose probably promotes structure-forming processes at the interface. This is in accordance with the data on callose-deficiency mutants (Dong et al. 2005; Nishikawa et al. 2005; You et al. 2010).

Spatial formation of the ring-like apertures is initiated during the late tetrad stage by invagination of the corresponding portions of the microspore surface (Fig. 9a, arrows). The sites which undergo invagination have been predestined: the first layer of the glycocalyx is completely absent in this site, and the second (inner) glycocalyx layer is much thinner than in interapertural sites (Fig. 12a, asterisks).

Distinctive features of organelles

The RER definitely plays an important role during the tetrad period. Its activity starts at an early tetrad stage when it participates in the synthesis of osmiophilic globules (Fig. 2a) and it continues to be intensively involved at the initiation of glycocalyx establishment (Fig. 3a, c) and during exine pattern establishment (by preventing the approach of Golgi vesicles to the plasma membrane between lens-like glycocalyx clusters; Figs. 4 and 6c). Curves of stacked RER cisternae occur through the volume of the microspore cytoplasm (Fig. 5d) and seem to participate in the synthesis and delivery of SP precursors to the glycocalyx at the stage of procolumella formation (Fig. 8a–d). Stacked RER is also abundant during the late tetrad stage (Fig. 9b).

Plastids with dark globules along the surface of the late tetrad microspores (Figs. 11d, 12a) and also in the tapetum

(Fig. 12b) are of particular note. Globules are located between the two restricting membranes of the plastids. Similar globules have been observed along the surface of double-membrane organelles (most probably a small generation of plastids) in *Chamaedorea microspadix* (Gabarayeva and Grigorjeva 2010), but in this species they are separated either by double membranes and located inside the deep invaginations of these organelles or they are located within the luminae of the two membranes (Fig. 6b in Gabarayeva and Grigorjeva 2010). In both species the role of these unusual plastids remains ambiguous. Our previous cytochemical data in *Chamaedorea* material provides some evidence that hypersynthesis of some lipoprotein substance (or nonspecific lipid transfer protein) takes place.

Needle-like crystals inside mitochondria during the early tetrad stage in *Passiflora* (Figs. 1e, 3a, arrows) are similar to those observed in *Chamaedorea* inside double-membrane organelles, where they form asterisk-shaped or needle-shaped structures (Fig. 6c–d). In both species the appearance of unusual plastids and needle-like crystals within mitochondria are evidently coupling processes.

Tapetum development

The parietal secretory tapetal cells during the early tetrad stage, as mentioned above, synthesize osmiophilic globules (Fig. 1b, c) and, as microspores, produce the glycocalyx (Fig. 7d), which however lacks the sites for SP accumulation. Later during the tetrad stage the tapetum still preserves its parietal position; its glycocalyx has the same radially oriented substructure as that of the microspores (Fig. 12b).

The underlying cause of development

In our earlier hypothesis (Gabarayeva and Hemsley 2006; Hemsley and Gabarayeva 2007) we suggested that the whole exine development in the periplasmic space represents the unfolding self-assembling sequence of successive colloid micellar mesophases (initiated by genomically determined physicochemical parameters) of glycoprotein surfactants (the main substances of the glycocalyx) and lipid surfactants including fatty acid and phenyl propanoid SP monomers (Gubatz et al. 1986; van Bergen et al. 1995; Hemsley et al. 1996b; Collinson et al. 1993; Wilmesmeier and Wiermann 1995; Kawase and Takahashi 1995; Niester-Nyveld et al. 1997; Meuter-Gerhards et al. 1999; Wiermann et al. 2001; Van Bergen et al. 2004; Grienberger et al. 2010) at increasing concentrations. The hypothesis was based on earlier self-assembly ideas (Hemsley et al. 1992; Gabarayeva 1993), on reconsideration of our results from the study of the pollen wall

ontogeny of a number of angiosperms and gymnosperms performed before 2006 and on experiments modelling artificial sporoderm (Hemsley et al. 1996a, 1998, 2000; Moore et al. 2009). The hypothesis was supported (Blackmore et al. 2007) and shared (Blackmore et al. 2010). Our further developmental studies (see the list of references) have provided additional evidence for this hypothesis. We observed the same sequence of micellar mesophase with small variations, including additional transitive mesophase stages, in all the species studied.

The observations on *Passiflora* during the tetrad period add to the previous ones and provide new evidence for the idea of exine development as a self-assembly sequence, unfolding in the periplasmic space. Figure 13 shows images of the main steps in exine development during the tetrad period (on the left) and corresponding micellar mesophases (on the right). The first accumulations seen on the plasma membrane of early postmeiotic microspores are osmiophilic globules with cogged edges. These are most probably very large spherical micelles with their typical “jagged” surface. They consist of diphilic molecules of a lipid surfactant with hydrophobic tails pointing inwards and hydrophilic heads poking out (“cogs”; Figs. 2b–f, 13a). These micelles coalesce and are gradually mixed with the newly appearing surfactant comprising glycoproteins delivered to the periplasmic space by the Golgi vesicles. A thin network of the glycocalyx appears. By this time the new self-assembling process—tensegrity—interferes in the process. Bundles of MF pull the plasma membrane inside at some points, resulting in the appearance of regularly disposed invaginations (Fig. 4). New portions of the glycocalyx appear inside these invaginations as microgranules resulting in a new generation of small spherical micelles (Figs. 5a–c, 13b). At the next developmental step, when the plasma membrane invaginations become wider and deeper, clusters of glycocalyx inside them have grown considerably by the addition of glycoproteins delivered to the periplasmic space (Fig. 6). At higher concentrations of this surfactant the next micellar mesophase appears. Cylindrical micelle tufts appear first as separate units (Figs. 7c, 13c, arrowheads), and later as whole clusters with tightly packed radially oriented units—the so-called “middle”, or hexagonal micellar mesophase. Then more glycoproteins, probably of slightly different chemical composition, are added onto the plasma membrane as the second generation of glycocalyx (glycocalyx-2; Fig. 8a). The latter has the same substructure (middle mesophase with radially oriented rod-like units), but the units are smaller in height and width, so the whole layer is denser (Fig. 8a). After the appearance of SP monomers (fatty acid and phenyl propanoid surfactants) in the periplasmic space, procolumellae appear on the framework of groups of glycocalyx-2 micelles tufts (Figs. 8c–f, 13d, arrowheads). At

the same time the glycocalyx-1 layer becomes sparse, and its substructure appears highly prominent. It appears as radially oriented cylinder-like units with a binder unit in the form of a spiral (Figs. 11a, b, 13e, f), which correspond to the rod-like core-binder fundamental model of exine units of Rowley (1990). The appearance of large tufts (supermicelles) in the glycocalyx-1 layer is in accordance with the statement of Ball (1994) that “when two or more similar micelles encounter each other they may merge”. The increasing concentration of hydrophobic SP monomers in the periplasmic space causes the inversion of normal cylindrical micelles (they turn inside out; Fig. 13e', e''), resulting in the appearance of an axial hydrophilic channel inside every procolumella (Fig. 8c–e). Thus, a “hollow” columella is an inverted supermicelle, with a hydrophilic interior allowing passage of water-soluble nutrients from the tapetum—a kind of a microchannel.

To summarize, our interpretation of the developmental events during primexine development in *Passiflora* reduces to the following micellar mesophases: large spherical micelles (osmiophilic globules) → small spherical micelles (microgranules inside invaginations) → cylindrical micelles → hexagonally packed cylindrical micelles—middle mesophase (glycocalyx-1) → second layer of middle mesophase (glycocalyx-2) → micelle tufts + SP monomers → procolumella + SP monomers → inversion of normal cylindrical micelles → procolumellae with an axial gap.

Conclusions

1. Prepattern structures comprise many osmiophilic globules with cogged surfaces which encrust the plasma membrane of the early tetrad microspores. These globules are probably large spherical micelles with this characteristic surface. Similar globules also cover the surface of tapetal cells. In both types of cells osmiophilic globules are synthesized by the SER.
2. Highly ordered pattern of the cytoskeleton elements (bundles of MF and MT, and also of RER cisternae, all of them arranged perpendicular to the plasma membrane) predestines the appearance of regularly located cup-like invaginations of the microspore plasma membrane. The process of counteraction of the cytoskeleton and ECM (= glycocalyx), a self-assembling tensegrity mechanism, assists in further primexine pattern formation.
3. Glycocalyx-1 appears inside the invaginations first in the form of microgranules, then as lens-like clusters of radially oriented rod-like units. Microgranules represent the generation of smaller spherical micelles, and rod-like units are the next self-assembling micellar

mesophase (cylindrical micelles), which later become tightly packed in a layer (middle, or hexagonal mesophase). These clusters of the first glycocalyx are responsible for the appearance of luminae of the future exine reticulum and later gradually disappear as a result of the loss of contact with the plasma membrane.

4. Glycocalyx-2 appears at the late tetrad stage as a continuous layer along the microspore surface. It also has a substructure of radially oriented units (micelles) and is a framework for the appearance of procolumellae: large and tectate ones of the future muri, and small and atectate of the future luminae. Gradual receptor-dependent SP accumulation causes first the formation of rod-like procolumellae, which later acquire an axial gap. This last phenomenon is easy to explain by inversion of normal cylindrical micelles to reverse ones, with a hydrophilic core. The latter functions as a channel for the passage of water-soluble nutrients species from the anther locus into the microspore cytoplasm. Such inversion at the late tetrad stage is a widespread feature of procolumellae in many species.
5. During the late tetrad stage portions of the microspore surface, predestined to be ring-like apertures, are invaginated.
6. Consideration of the detailed developmental stages in the course of primexine formation as a self-assembling micellar sequence reveals the appearance of three first mesophases in this sequence: spherical, cylindrical and middle (layers of hexagonally packed cylindrical) micelles. Reiteration of mesophases over time is observed: spherical micelles occur twice (the first generation disintegrate), and middle micelles appear twice, bringing about the formation of the first and the second glycocalyx layers. Another self-assembling process—tensegrity, based on relationships between cytoskeleton and ECM (glycocalyx)—interferes in primexine development, assisting pattern formation.

Acknowledgments This work was supported by grant RFBR No. 11-04-00462a to Nina Gabarayeva. We thank our engineer Peter Tzinman for assistance with the Hitachi H-600 transmission electron microscope. We particularly thank Bruce Sampson for correcting the English text. This study on *Passiflora* exine development was reported at IPC XIII 2012, at the symposium SS10 “Exine development and pattern formation, unifying ultrastructural and genetic approaches” (28 August, Tokyo, Japan) which was dedicated to the memory of the outstanding palynologist John R. Rowley. We cordially thank the co-organizers of the symposium Steve Blackmore and Michael Hesse who did much in preparing this meeting. We heard seven interesting talks, including the introduction by Steve Blackmore, reviewing the past, present and future of exine development studies, and his concluding remarks. The studies, reported at the symposium showed that ideas of Rowley continue to inspire new investigations of different aspects. John Rowley did not set up official “schools of thought”, but such a school has appeared spontaneously, without his special effort.

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