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Pollen exine development precedes microtubule rearrangement in *Vigna unguiculata* (Fabaceae): a model for pollen wall patterning

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Summary. Although patterns on pollen exines are highly conserved, heritable traits, there is no prevailing explanation for control of pattern development. In *Vigna unguiculata* (Fabaceae), the exine reticulum is unusually coarse so that development of the reticulum can be followed by light microscopy. Developing exine patterns were compared with the arrangement of microtubules in the microspore using immunofluorescence labeling of microtubules. Exine pattern developed in microspores at stages with a regular microtubule pattern. At later stages of exine formation, microtubules were arranged in patches under the lumina of the reticulum. We conclude that microtubules do not determine exine pattern. The developing exine appears to rearrange microtubules. We interpret this as evidence for the self-patterning of exine based on intrinsic properties of the matrix between the microspore and the callose wall.

Keywords: Exine; Extracellular matrix; Microtubules; Pattern formation; Pollen.

Abbreviations: DIC differential interference contrast; ECM(s) extracellular matrix(ces); MT(s) microtubule(s); PBS phosphate buffered saline; SEM scanning electron microscopy.

Introduction

Pollen grains are enclosed by multilayered cell walls that are organized in complex, heritable patterns (Erdtman 1952). Related plants usually have similar exine patterns. Exine sculpturing is among the most stable morphological traits expressed by plants. The exine (outer cell wall layer) is composed of sporopollenin, a highly resistant hydrocarbon material (Wiermann and Gubatz 1992, Southworth 1990). Exine patterns include radiating elements with acute or bulbous tips, sometimes linked into reticulate patterns or completely covered with a tectum (Erdtman 1952). The radiating rods may be linked at their bases and sometimes at an intermediate level. Other patterns are based on non-homogeneous interconnected elements. Pollen exines are initiated as patterned primexines composed of proteins and polysaccharides which later become impregnated with more resistant materials (Heslop-Harrison 1963). Exine patterns in pollen develop after meiosis while the tetrad of haploid microspores is surrounded by a callose cell wall which holds the tetrad together and separates individual microspores (for reviews, see Blackmore and Barnes 1990, Takahashi and Skvarla 1991). Each microspore lies in a cavity surrounded by callose. The exine is initiated and patterned on the cell surface within the cavity.

The mechanisms that determine pollen exine pattern are unknown (Takahashi 1989). From developmental studies of numerous taxa using electron microscopy, no organelle configuration has been observed that would indicate how the pattern is generated (Dickinson and Sheldon 1984). Recently, a correlation was observed between the configuration of cortical MTs and exine pattern in *Vigna vexillata* (Muñoz et al. 1995). In *Vigna*, the exine is coarsely reticulate, consisting of raised ridges or muri surrounding thin-walled spaces or lumina (Pérez-Muñoz et al. 1993 a, b). The reticulum in *Vigna* is unusual in that it is a

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mass of solid non-homogeneous elements instead of radial columns (Horvat and Stainier 1979; Stainier and Horvat 1978 a, b; Pérez-Muñoz et al. 1993 a, b). Muñoz et al. (1995) observed patches of MTs under the lumina of the exine reticulum on immature microspores in tetrads. This suggested a causal relation between MT pattern and exine pattern.

These results were surprising because MTs did not correlate with exine pattern in developing microspores of *Lilium* (Dickinson and Sheldon 1984; Sheldon and Dickinson 1983, 1986). Also, colchicine applied to buds of *Lilium* and *Tradescantia* produced no major changes in exine patterns, although MTs were absent following colchicine treatment, and the shape of pollen was altered (Heslop-Harrison 1971, Sheldon and Dickinson 1986, Tiwari and Gunning 1986, Tiwari 1989).

However, plant cell walls may be considered homologous to aminal cell ECMs (Wyatt and Carpita 1993). In animals, the ECM-cyskeleton scaffold, linked via the cell membrane, coordinates cell differentiation (Adams and Watt 1993, Ingber 1993, Wang et al. 1993, Wyatt and Carpita 1993). Additional evidence is accumulating for an interaction between plant cell walls and the cytoskeleton that leads to developmental change (Wyatt and Carpita 1993).

We analyzed the spatial relationships and developmental timing of MT and exine patterns in *Vigna* to determine whether one forms before the other or whether the patterns occur simultaneously. Based on our results, we propose a model for control of pollen wall pattern formation involving interactions of ECM, plasma membrane and intracellular pressure. Our goal is to formulate testable hypotheses about the mechanism of exine pattern formation.

Materials and methods

Plants of cowpea, *Vigna unguiculata* (L.) Walp. (Fabaceae), were grown in a greenhouse. Mature flowers and fruits were removed regularly to prevent bud abortion. Immunocytochemical methods were adapted from Baskin et al. (1992), Brown et al. (1989), Smith-Huerta and Jernstedt (1989), Wick and Duniec (1986), Tiwari and Polito (1990), and Wick et al. (1985). Anthers were dissected from buds and fixed in 4% para-formaldehyde buffered in 50 mM PIPES, pH 6.8, containing 5 mM EGTA, 5 mM $MgSO_4$ (PEM) plus 5% dimethylsulfoxide. One anther from each flower was squashed to determine developmental stage. Fixed anthers were chopped with a razor blade to release tetrads; material was sieved to remove anther debris. Thirty minutes after the last anther material was put into fixative, tetrads were washed with PEM and incubated in 2% yeast lytic enzyme (a recombinant β-1,3-glucanase containing no protease; ICN Biochemicals) in PEM for 30 min at room temperature (22–26 °C) with rotation.

Immunofluorescence labeling was carried out in 1.5 ml microcentrifuge tubes. Tetrads were washed with PBS containing 1% Triton-X 100; incubated for 1 h at 24 °C in monoclonal mouse anti-chicken β-tubulin IgG (Amersham) diluted 1 : 200 with PBS; washed with PBS; incubated in goat anti-mouse IgG-FITC (Amersham) diluted in 1 : 50 with PBS, for 1 h at 37 °C; washed with PBS and mounted in moviol with n-propyl gallate. Controls included omitting the first or second antibody. Slides were examined on an Olympus BHS microscope with DIC optics and epifluorescence. Selected cells were observed with a Biorad MRC 600 Scanning Confocal Imaging System on an Olympus BH2-RFCA microscope with a ×60 oil immersion lens (N.A. 1.4). Optical sections were observed at 1 μm intervals.

For SEM, mature pollen was dusted onto sticky tape on a stub, sputter-coated with gold, and examined in a Hitachi S-2100 SEM.

Results

The exine of *Vigna unguiculata* was reticulate with raised muri and irregular lumina (Fig. 1). This pattern formed during the tetrad stage while four meiotic products were encased in the callose cell wall. Three distinct stages of pollen wall development were rec-

Fig. 1. SEM of mature pollen of *Vigna unguiculata* showing reticulate exine with raised muri (*M*) encircling flattened lumina (*L*); *A* apertures at apices of triangular pollen grain. ×600

Fig. 2–7. Unpatterned microspores. ×1,500

Fig. 2. Microspore surface of fresh tetrad; smooth, no evidence of reticulate pattern (DIC)

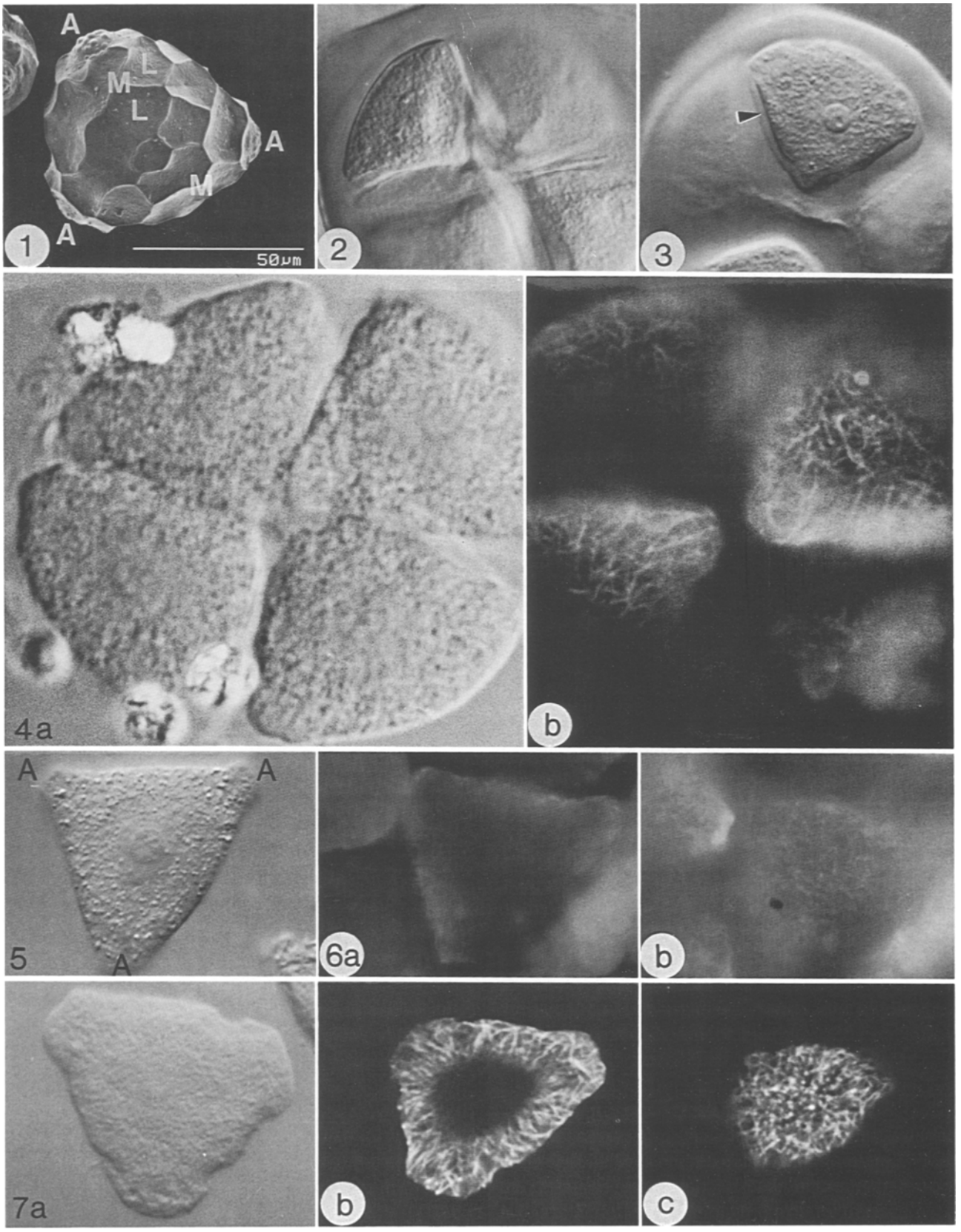
Fig. 3. Microspore separated from inner surface of callose (arrowhead), possibly indicating secretion of unpatterned primexine matrix. Cortical organelles are dispersed without pattern; nucleolus visible (DIC)

Fig. 4. a Flattened tetrad, surface uneven, but not reticulate. **b** MTs curve at cell surface and parallel the surface in coarse bundles; no reticulate pattern

Fig. 5. Microspore released from tetrad by pressure (DIC). Appertures (*A*) bulged; surface unpatterned

Fig. 6. a MT bundles thinner and more evenly spaced than in Fig. 4 b. **b** MT arrangement in cortical cytoplasm does not correlate with future reticulate pattern

Fig. 7 a–c. Transitional stage of unpatterned microspore. **a** Microspore surface with sparse depressions. **b** MTs radiate from nucleus and curve under the cell surface (confocal image). **c** MTs dispersed evenly through cortical cytoplasm. No MT pattern correlates with or precedes a reticulum (confocal image)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ognized during the tetrad period: (1) unpatterned microspore corresponding to stages 2 and 3 of Pérez-Muñoz et al. (1993 a, b); (2) patterned surface corresponding to stage 4 of Pérez-Muñoz et al. (1993 a, b); and (3) patterned primexine corresponding to stage 5 of Pérez-Muñoz et al. (1993 a, b). Within each stage were early and late forms. The stages differed in surface contours, in MT patterns, and in degree of exine development. In all three stages, the microspores remained within cavities surrounded by callose.

Unpatterned microspore

A stage existed, after completion of meiosis, during which no evidence of the future exine pattern was detected. The microspore surface was expanded (Figs. 2–6). Microspores were triangular in profile and flattened. In some microspores, observed fresh with DIC optics (Fig. 3), a possible primexine matrix was detected, corresponding to stage 3 of Pérez-Muñoz et al. (1993 a, b). After treatment with β-1,3-glucanase, this layer was not visible (Fig. 4). MTs were arranged in two types of patterns. One had coarse MT bundles criss-crossing the cortical cytoplasm with uneven spacing (Fig. 4 b). At a slightly later stage, with shallow, flat-bottomed depressions in the surface, MT bundles were finer and more regularly spaced (Figs. 6 a, b and 7 b, c).

Patterned surface

The future reticulate pattern first appeared as ridges and depressions on the microspore surface (Figs. 8–12). The plasma membrane protruded at sites of future muri and was depressed at sites of future lumina. No reticulate primexine was observed by DIC optics or by autofluorescence. The internal surface of the callose cavity remained smooth and did not assume the contours of the microspore surface. MT pattern resembled that of the unpatterned microspores with coarse MT bundles (Fig. 11 b). Late in the surface patterned stage, the primexine was initiated (Fig. 12). At this stage, the surface remained patterned with deep depressions. A thin tracing of reticulate primexine formed on the microspore surface and MTs radiating from the nucleus occupied positions under the protrusions at the site of murus formation (Fig. 12 b, c).

Patterned primexine

Following the patterned surface, a tracing of reticulate primexine developed on protruding regions of the plasma membrane (Figs. 13–16). The primexine autofluoresced pale yellow with FITC filters and was visible with bright field and DIC optics (Fig. 13). Three patterns of MTs were found in microspores with patterned primexines: (1) a coarse weftwork of MTs throughout the cortical cytoplasm similar to that of unpatterned microspores (Figs. 14 b, c and 16 b–d); (2) a rim of MTs underlying the edges of lumina (Fig. 15 d) and (3) patches of MTs underlying the centers of lumina (Figs. 14 d and 15 b, c). On some microspores, two different patterns were observed on opposite sides. In one microspore, MTs were arranged in a weft on one side of the cortical cytoplasm, and in patches under the lumina on the other side (Fig. 14). In another, MTs formed rims under the lumina on one side, and formed patches under lumina centers on the other (Fig. 15).

Discussion

Developmental timing of exine and microtubule patterns

Patches of MTs in the lumina of the reticulum do not constitute a pre-pattern for the exine because the patches were observed *after* the exine pattern was evident on the microspore surface. Although MTs are present in developing microspores, their arrangement does not coincide with exine pattern until that pattern

Figs. 8–12. Microspores with patterned surface. ×1,500

Figs. 8–10. Unfixed microspores from tetrads (DIC)

Fig. 8. Microspores with small, shallow surface depressions slightly more patterned than Fig. 7 a

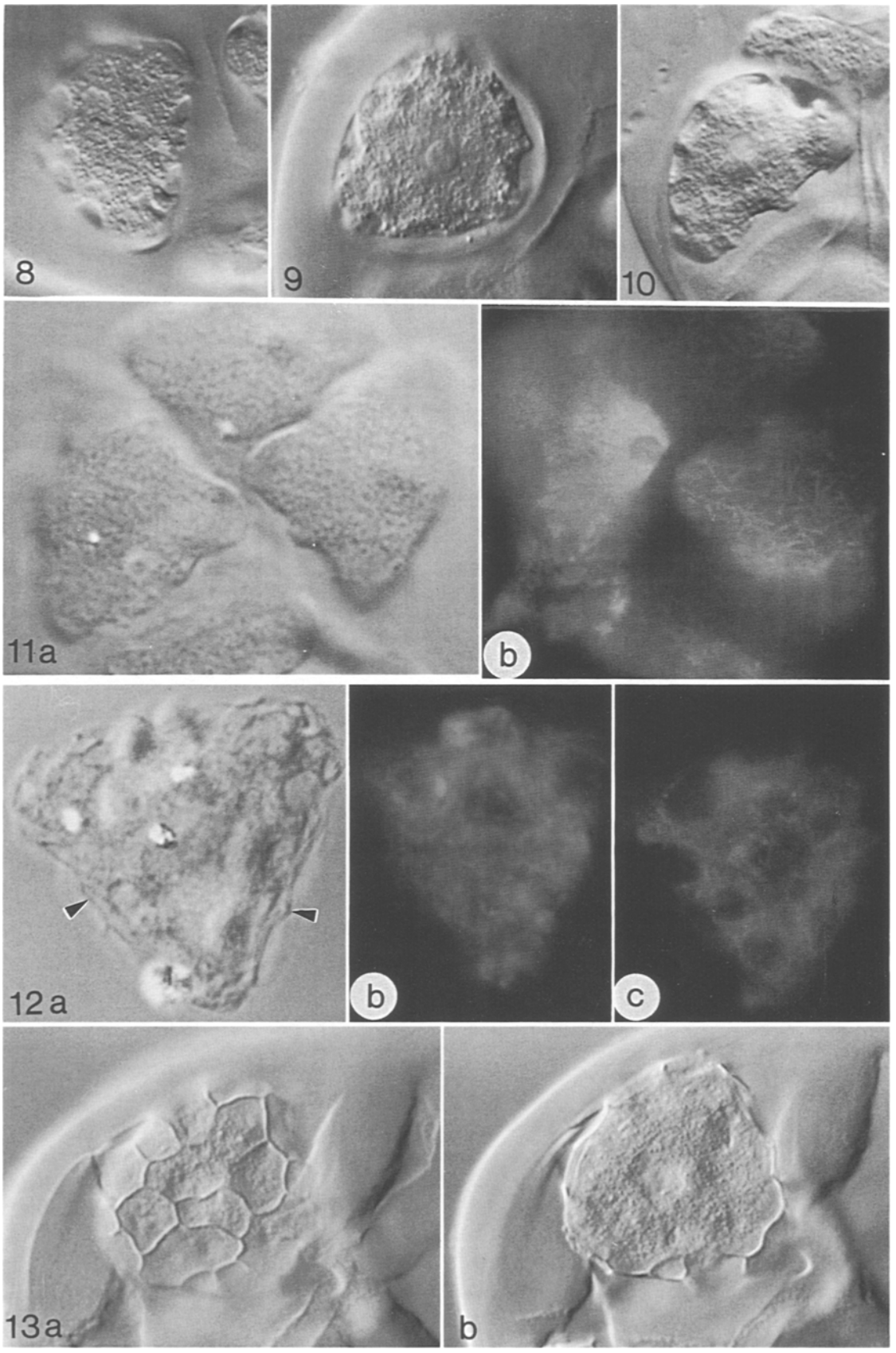
Fig. 9. Regular depressions in microspore surface coincide with future lumina of reticulum

Fig. 10. Broad, deep depressions in surface coincide with future lumina of reticulum

Fig. 11. a Flattened tetrad. **b** MT pattern not reticulate; coarse MT bundles similar to those in unpatterned microspores (Fig. 4 b)

Fig. 12. a Transitional stage with thin primexine (arrowheads); larger surface depressions. **b** One surface of microspore with some lumina free of MTs. **c** Opposite surface of microspore with MTs surrounding lumina

Fig. 13 a, b. Microspore with thin patterned primexine (DIC). ×1,500. **a** Surface pattern of primexine reticulum. **b** Microspore expanded, depressions in surface shallower than those of surface patterned microspores, slightly thicker muri and smoother lumina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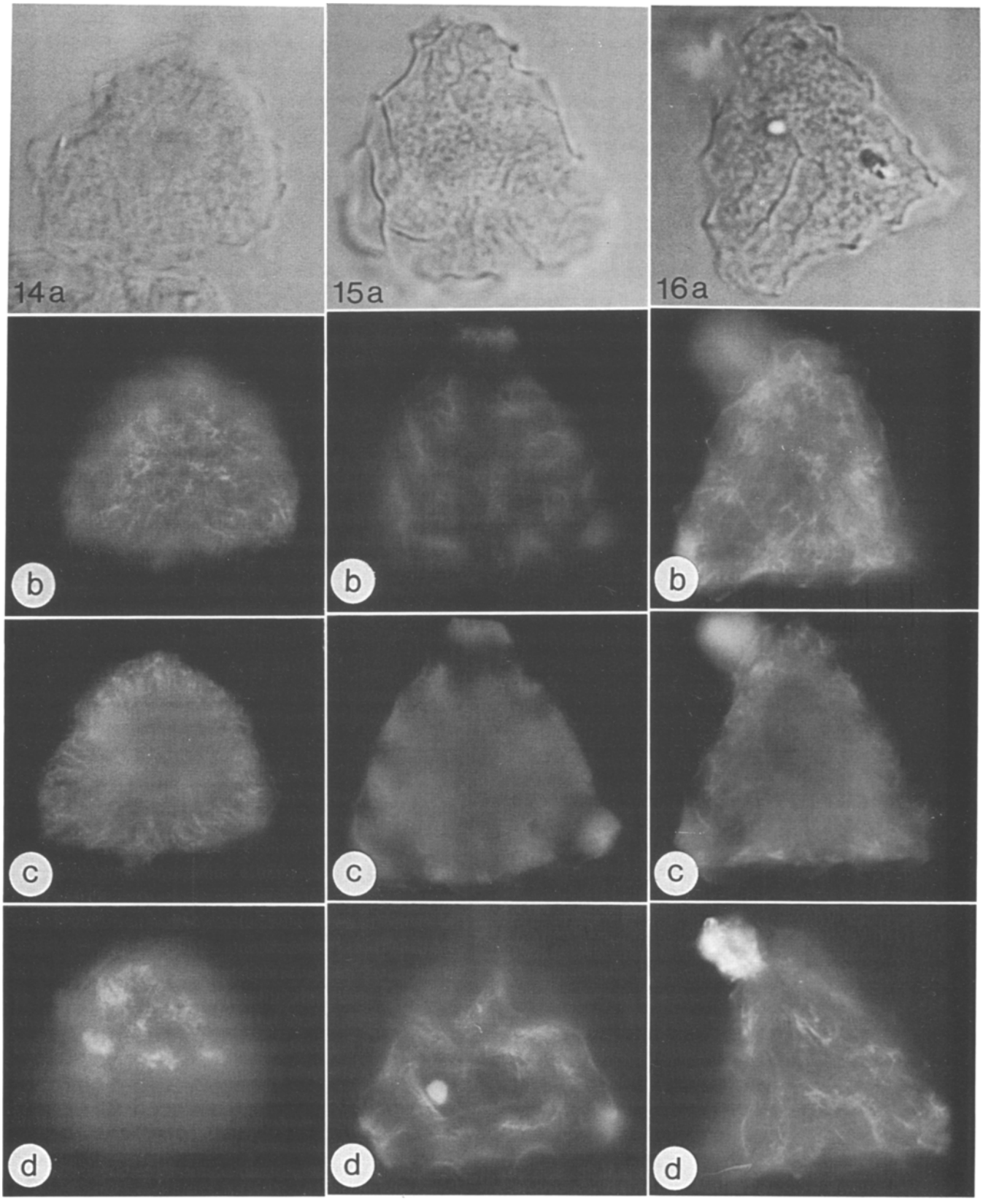
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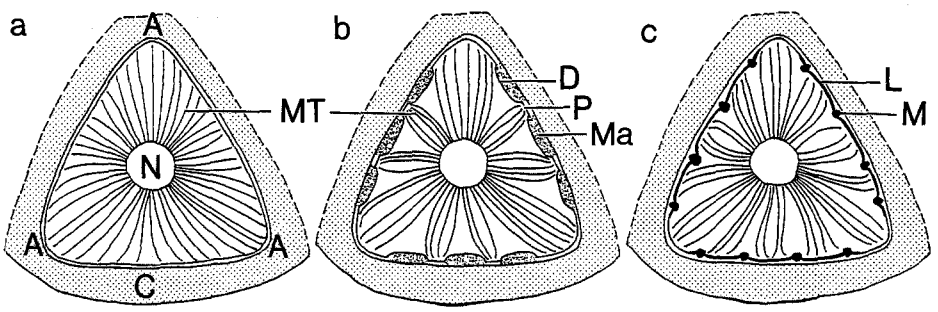

Fig. 17. Diagram of hypothetical relationship between exine patterning stages and MT pattern. One microspore of each tetrad is shown surrounded by the callose jacket (*C*). Apertures (*A*) form at the apices of the triangular microspore. **a** Unpatterned surface. Each microspore fills its cavity in the callose. No matrix material has been secreted; MTs radiate from nucleus (*N*) with no regular pattern. **b** Patterned surface. Secretion of primexine matrix (*Ma*) creates a mass of material forming a depression (*D*) in the surface of the microspore. Cell surface protrusions (*P*) occur at sites of future muri; depression at sites of future lumina. MTs extend into protruding parts of the cell. **c** Patterned primexine. Development of primexine reticulum causes the former protrusions to indent at developing muri (*M*). MTs now extend to regions under lumina (*L*)

has been established. MT pattern does not prefigure exine pattern.

This explanation is consistent with observations that colchicine does not change the basic exine surface pattern (Heslop-Harrison 1971, Sheldon and Dickinson 1986, Tiwari and Gunning 1986, Tiwari 1989). These observations verify the pattern of MT patches under exine lumina (Muñoz et al. 1995), but show clearly that patches of MTs do not occur until well after exine pattern has been established.

Although there is considerable variation in MT pattern, especially at later stages of exine patterning, the coincidence of MT pattern and exine pattern suggests some relationship. While it is clear that MTs were not responsible for patterning the exine, the reverse cannot be ruled out.

Cellular tensegrity

One possible explanation for the relationship between extracellular matrix and cytoskeleton in developing pollen grains is based on the cellular tensegrity model derived from architectural designs of Buckminster Fuller and applied to cell structure by Ingber (1993). According to this model, which has been invoked to explain how adhesion to an external substratum can influence cell differentiation, mechanical forces applied by the ECM connected to a substratum are detected by the cell membrane, transduced to the cytoskeleton, and possibly transmitted, as intracellular signals, to the nucleus (Wang et al. 1993). According the cellular tensegrity model, the cytoskeleton is considered as a whole, with semi-rigid MTs linked by a lattice of contractile microfilaments and intermediate filaments, with microfilaments connected to the cell membrane. A force or pressure exerted at one or a few points on the cell surface results in a rearrangement of the entire cytoskeleton (Sims et al. 1992, Adams and Watt 1993). Integrated changes in cell shape result from a stimulus by the ECM acting through the membrane to the cytoskeleton. The signal for change comes from the ECM in the form of pressure or tension; the response is made by the cytoskeleton. Cellular tensegrity does not require a direct molecular connection between the ECM and the cytoskeleton (Ingber 1993, Wang et al. 1993).

The components necessary for cellular tensegrity are present in developing microspores. An ECM is present (the primexine matrix); there is a rigid substratum (the callose wall) and a cell membrane around a cell with a cytoskeleton.

Model for mechanism of exine patterning

We envision the following sequence of events. In the unpatterned microspore, MTs originating on the

Fig. 14–16. Microspores with patterned primexine. ×1,500

Fig. 14. **a** Primexine around microspore. **b–d** Through-focus series. **b** At one surface, MTs in weft pattern. **c** MTs radiate, curving near surface. **d** Opposite surface with MT patches under lumina of reticulum

Fig. 15. **a** Exine slightly thicker than in Fig. 14. **b–d** Through-focus series. **b** At one surface, MTs form patches under lumina of reticulum and under aperture. **c** MTs under lumina of reticulum. **d** Opposite surface with MTs curving around edge of lumina

Fig. 16. **a** Primexine reticulum. **b–d** Through-focus series. **b** Radiating bundles of MTs. **c** Optical cross section; radiating MTs, curved near cell surface. **d** Some MTs form clusters under lumina

nuclear envelope radiate into the cortex. Cells are under positive pressure and fill the cavity within the callose jacket. Then, primexine matrix is secreted by the endomembrane system of the microspore (stage 3 in Pérez-Muñoz et al. 1993 a, b). The matrix is secreted uniformly without regard for future pattern except for its absence at apertural sites. Because of the hydrated nature of the ECM and sustained osmotic pressure, the cell surface is under pressure within the fixed volume of the cavity (Fig. 17 a). MTs do not shorten, but bend or move to areas where the plasma membrane protrudes, thus allowing greater indentation of the plasma membrane in other regions now free of MTs (Fig.17 b). As the matrix changes and primexine forms at protrusions, the protrusions become more rigid (Fig. 17 c). The former depressions become relatively more flexible parts of the cell surface and bulge out. MTs again move by physical forces to more stable positions, now in the middle of lumina (Fig. 17 c).

According to this explanation, exine pattern is generated by physical properties of (1) the callose shell, (2) the matrix and primexine, and (3) conditions in the microspore that generate osmotic pressure and cytoskeletal tension. We interpret MT pattern as a response to changing physical forces. The pattern is a spontaneous, stabilizing one that occurs in local areas. There is no cytoplasmic preplan, rather, the pattern formed is a response to tensile and rigid properties of the cytoskeleton and to osmotic pressure in the microspore, balanced against the pressure and volume of the newly secreted matrix.

Evidence in support of the model

This model is consistent with observations that some exine patterns are highly regular geometric arrays while others are fractal patterns, both of which are spontaneous responses to physical conditions and constraints, and not under the direction of a blueprint (Van Uffelen 1991).

The callose cell wall is a common feature of pollen-bearing plants. In *Ipomoea*, with large spherical pollen, the callose cavity bears the imprint of the primexine matrix; whether this is by accretion or indentation is unknown (Waterkeyn and Bienfait 1970). However, in *Vigna* and in *Caesalpinia* (Takahashi 1989), no imprint of the exine reticulum was observed. Callose is not considered to be the organizer for the wall pattern, but it may reflect patterning that occurred during secretion of the primexine matrix. In transgenic tobacco, in which premature callase (β-1,3-glucanase) activity destroys callose before exine patterning, the exine pattern was abnormal (Worrall et al. 1992). These observations underscore the existence of pressure within the cavity, the role of callose as a physical restraint, and a restraining role for the callose wall in pattern formation. The presence of primexine matrix is also a common feature of pollen development. Its role in pattern formation is not known. On exines of *Cucurbita pepo* pollen, there are two sets of spines, large conical ones and smaller cyclindrical ones (Nepi and Pacini 1993). These form in sequence, each within its own layer of primexine matrix (M. Nepi and E. Pacini unpubl.). This suggests two patterning events with similar mechanisms. According to our hypothesis, the primexine is a key organizer of pattern formation. Pattern differences among taxa could reflect the quantity of primexine material and its qualities particularly its degree of hydration and such properties as compressability, density, plasticity, and fluidity. Experiments that could determine differences in primexines would be valuable. These might include antibodies to primexine and possibly other cytochemical stains to compare ECM properties of related taxa with different exine patterns.

Variants of exine pattern among closely related taxa would be useful. These might be from mutations, either induced or spontaneous, from species with dimorphic pollen, or from related species with divergent pollen exine patterns.

MTs are present in developing microspores, but their role in the patterning of exine is uncertain. They are capable of exerting pressure and of responding to stress and compression and to changes in the extracellular matrix (Bajer et al. 1982, Ingber 1993, Williamson 1991). Plant microtubules are capable of rearrangement (Palevitz 1993). The hypothesis presented here suggests that further experiments involving drugs that manipulate MTs will not be productive in determining how exines are patterned or in changing the pattern.

Experimental manipulation of exine would provide strong support for the hypothesis. In vitro culture of developing tetrads under conditions that lead to pollen formation, rather than to embryogenesis, will be valuable. For example, such an in vitro system could be used to test the effect of internal cell pressure on exine patterning.

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