Sex Plant Reprod (1995) 8:147–151

ORIGINAL PAPER

Carlos A. P. Muñoz · Barbara D. Webster
Judith A. Jernstedt

Spatial congruence between exine pattern, microtubules and endomembranes in *Vigna* pollen

Received: 7 October 1994 / Revision accepted: 10 November 1994

Abstract The role of microtubules and endomembranes in pollen wall pattern formation in *Vigna vexillata* L. was examined using fluorescence laser scanning confocal microscopy. Indirect immunofluorescence using anti-β-tubulin antibodies revealed that the arrangement of the cortical microtubular cytoskeleton in microspores resembled the reciprocal of the reticulate ektexine ornamentations of mature *V. vexillata* pollen. Patches of microtubules in cortical cytoplasm corresponded in location with the lumina of the exine reticulum and with apertural sites. Microtubules were absent from cytoplasm under muri (ridges) of the exine reticulum. Labeling of microspores during the mid-tetrad stage with the endomembrane-specific fluorochrome $DiOC_6$ produced a pattern similar to that of the microtubules; i.e., $DiOC_6$ staining was localized in cytoplasm underlying lumina and absent from cortical cytoplasm underlying sites of muri. This report represents the first observation of congruence of the pattern of occurrence of any subcellular organelles with exine pattern and, in particular, the congruence of both microtubules and endomembranes in cortical cytoplasm with the lumina of the reticulate exine.

Key words Microtubules · Cytoskeleton · Endoplasmic reticulum · Pollen · Exine · Pattern formation · *Vigna vexillata* · $DiOC_6$

Introduction

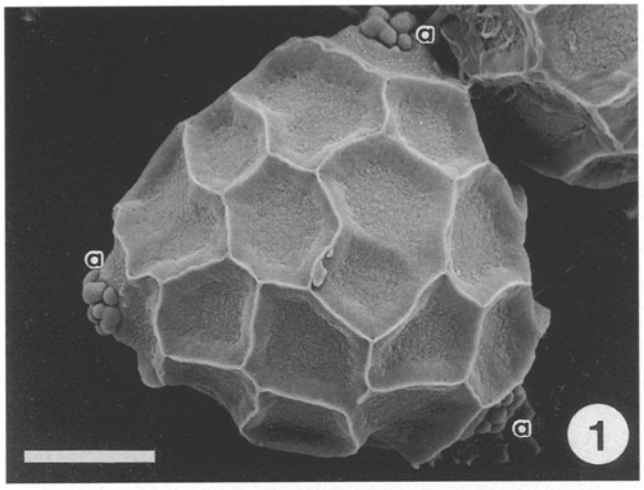
Angiosperm pollen grains show extraordinary diversity in patterns of wall sculpturing and structure. Additionally, pollen exine wall ornamentation is among the most stable of morphological traits expressed in plants. Although the control of exine patterning (specifically ektexine patterning) has been the subject of debate and research for decades, there exists no prevailing hypothesis on the origin of pollen wall pattern. Wall pattern has been suggested to be under gametophytic, sporocytic, or sporophytic control; the result of pre-patterning events; and the result of purely physical phenomena (Heslop-Harrison 1971; Sheldon and Dickinson 1983; Dickinson and Sheldon 1984; Skvarla and Rowley 1987; Blackmore and Barnes 1990; van Uffelen 1991). Although various cytoplasmic organelles and constituents are known to change in abundance and location throughout microsporogenesis and pollen maturation, none has yet been shown to exhibit a pattern that could be held responsible for development of the exine pattern. Thus, ample opportunity exists for comparative, developmental and experimental studies of pollen wall patterning to begin to elucidate possible mechanisms for this important and poorly understood event.

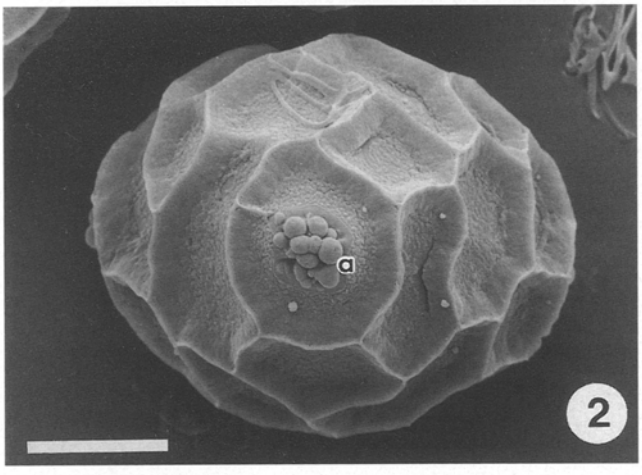
In pollen of *Vigna vexillata* L. (Fabaceae), a wild relative of cowpea, the exine pattern is an unusually coarse reticulum (Pérez-Muñoz et al. 1993a,b). The reticulum is formed of raised walls (muri) surrounding irregular spaces (lumina). The structure of the reticulum in pollen of *V. vexillata* differs from that of the more common reticulate pattern as seen in *Lilium* (Dickinson 1970) or in other genera of the Fabaceae such as *Caesalpinia* (Takahashi 1989) and *Poinciana* (Rowley and Skvarla 1987). In the latter taxa, muri are subtended by discrete columns (columellae), while in *V. vexillata* the walls are solid and appear granular in thin sections (Pérez-Muñoz et al. 1993a,b). Because granular construction is simpler than the columellate type, involving the patterning of fewer elements, and because the reticulum is coarser, we suggest that the processes involved in determination and development of this reticulate exine pattern may be easier to identify and interpret in *Vigna* than in other more complex or finely ornamented grains.

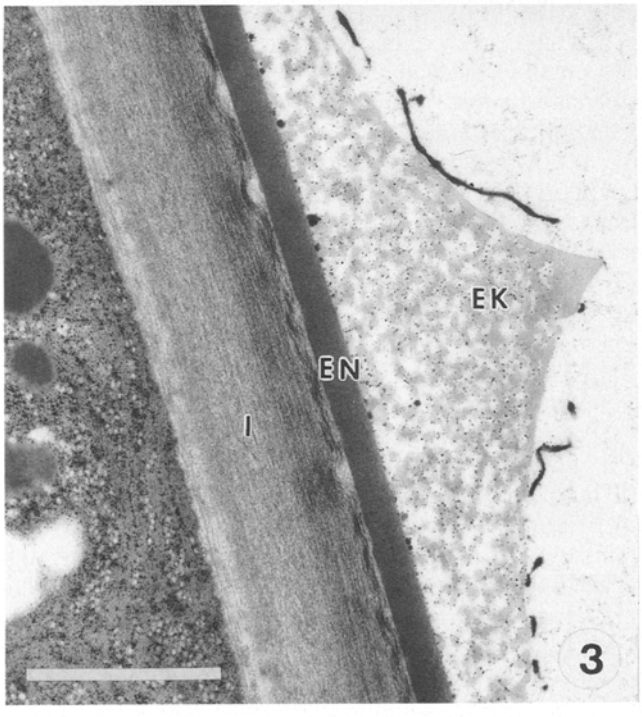
Using indirect immunofluorescence and fluorescent dyes and laser scanning confocal microscopy, we examined microtubule organization and arrangement of endomembranes in the cytoplasm of microspores of *V. vexill-*

C. A. P. Muñoz[1] (✉) · B. D. Webster · J. A. Jernstedt
Department of Agronomy and Range Science,
University of California, Davis, CA 95616, USA

Present address:
[1] Department of Biology, University of Puerto Rico, Mayagüez, PR 00681, Puerto Rico







Figs. 1, 2 Scanning electron micrographs of acetolyzed mature pollen of *Vigna vexillata*. **1** Polar view showing three apertures (*a*) arranged on the equator. Exine pattern is coarsely reticulate with raised muri surrounding polygonal lumina. *Bar* 20 µm. **2** Equatorial view. An operculum composed of irregular globules covers the aperture (*a*). *Bar* 20 µm

Fig. 3 Transmission electron micrograph of mature pollen wall showing inner microfibrillar intine (*I*), darkly staining endexine (*EN*), and granular ektexine ridge (*EK*). *Bar* 1 µm. (Figs. 1–3 reprinted from Pérez-Muñoz et al. 1993a)

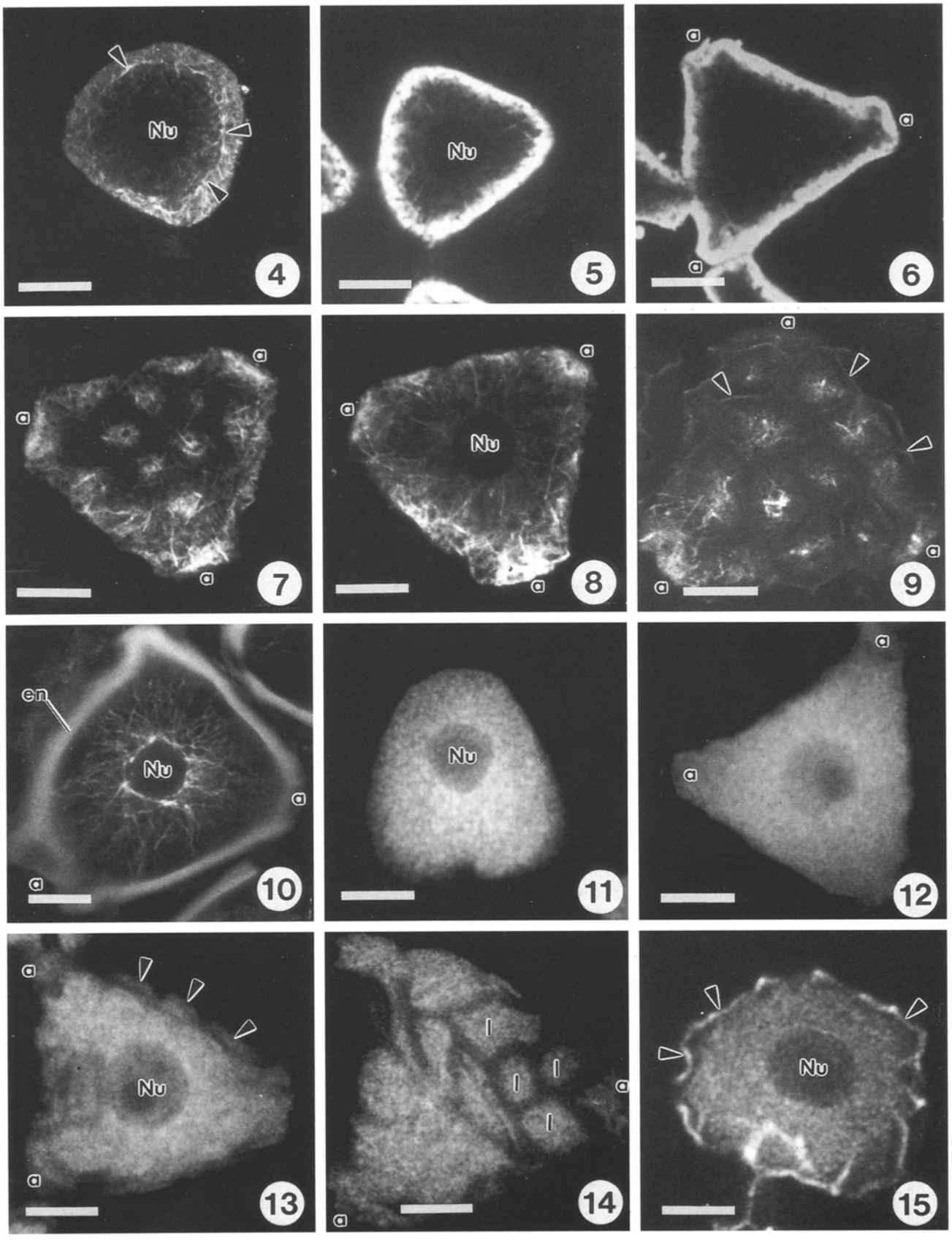
ata during development, at the time the reticulate pattern is initiated and established. Our results show congruence of the pattern of cortical microtubules and endomembranes with pollen exine pattern.

Materials and methods

Vigna vexillata L. (Fabaceae) cuttings from a single plant were grown to reproductive maturity in the greenhouse. Anthers dissected from flower buds were fixed in 4% paraformaldehyde in 10 mM PIPES buffer with 3 mM EGTA, pH 7.0, for 2 h (Smith-Huerta and Jernstedt 1989). Fixation was followed by three rinses

Figs. 4–10 Indirect immunofluorescent labeling of microtubules in microspores of *Vigna vexillata*. **4** In a young microspore, concentric microtubules (arrowheads) surround the nucleus (*Nu*); radial microtubules extend from the nuclear surface through cortical cytoplasm to the cell surface. *Bar* 10 µm. **5** Tangential optical section through cortical cytoplasm of young microspore at stage of protrusion of apertural sites. Microtubules radiate from the nucleus (*Nu*). Increased tubulin signal in cell cortex relative to Fig. 4. *Bar* 10 µm. **6** Median optical section of Fig. 5. Diffuse tubulin fluorescence occurs throughout the cortical cytoplasm including region under the apertures (*a*). *Bar* 10 µm. **7** Tangential optical section through cortical cytoplasm of microspore at onset of development of patterned primexine. Patches of microtubules coincide with lumina of the future reticulum and with apertures (*a*). *Bar* 20 µm. **8** Median optical section of Fig. 7. Patches of microtubules coincide with apertural sites (*a*) and with sites subsequently occupied by lumina of the patterned exine. Microtubules radiate from the nucleus (*Nu*) to the cortical cytoplasm. *Bar* 20 µm. **9** An older microspore within the tetrad shows autofluorescent primexine ridges (*arrowheads*). Congruence between lumina of reticulum and microtubule patches evident. *a* Apertures. *Bar* 10 µm. **10** A free microspore released from the tetrad. Microtubules are radially oriented from nucleus (*Nu*) to cell cortex. The endexine (*en*), an inner continuous exine layer covering the apertures (*a*), is autofluorescent. *Bar* 10 µm

Figs. 11–15 $DiOC_6$ labeling of endomembranes in microspores of *V. vexillata*. **11** Prepattern microspore with $DiOC_6$ fluorescence in a granular pattern throughout cytoplasm. Compare with Fig. 4. *Nu* Nucleus. *Bar* 10 µm. **12** Microspore at stage of protrusion of apertural sites (*a*). Compare with Fig. 6. $DiOC_6$ fluorescence remains granular throughout the cytoplasm. *Bar* 10 µm. **13** Median optical section of microspore at onset of development of the reticulate primexine. Compare with Fig. 8. $DiOC_6$ fluorescence is patchy in cortical cytoplasm and coincides with future lumina (*arrowheads*). *Nu* Nucleus, *a* aperture. *Bar* 10 µm. **14** Tangential optical section of Fig. 13. Patches of $DiOC_6$ fluorescence coincide with positions of lumina (*l*) and with apertures (*a*). Compare with Fig. 7. $DiOC_6$ also labels membranes throughout the cytoplasm. *Bar* 10 µm. **15** Microspore in late tetrad stage. Patterned exine (*arrowheads*) autofluoresces. Compare with Fig. 9. A granular $DiOC_6$ signal is found throughout cytoplasm, but not in the nucleus (*Nu*). *Bar* 10 µm


Nu
4
Nu
5
a
a
a
6
a
a
a
7
a
Nu
a
a
8
a
a
a
9
en
Nu
a
a
10
Nu
11
a
a
12
a
Nu
a
13
I
I
I
I
a
14
Nu
15

in the buffer and in phosphate-buffered saline (PBS). Pollen grains were then dissected from the anthers, sandwiched between coverslips, frozen in liquid propane, and freeze-fractured by prying apart the coverslips while frozen (Tiwari and Polito 1990). Cells were attached to coverslips by drying at 37°C for 2 h, rehydrated for 15 min in PBS, and incubated overnight in mouse monoclonal IgG to chick brain β-tubulin (Amersham) diluted 1:200 in PBS containing 1% Triton-X 100. After three rinses in PBS, cells were incubated 2 h in darkness in FITC-labeled goat anti-mouse IgG (Amersham) diluted 1:60 with PBS. Following repeated rinsing in PBS, cells were mounted in moviol with n-propyl gallate added to reduce fluorescence fading (Wick et al. 1985). Slides were examined with an Olympus BHS epifluorescence microscope, and with a BioRad MRC 600 laser scanning confocal imaging system. Optical sections were observed at 1 μm intervals, and photographs were taken from the confocal scope display monitor. Controls omitting primary, secondary or both antibodies showed no nonspecific labeling and allowed recognition of autofluorescence at later stages of wall formation.

For analysis of endomembranes by fluorescence microscopy, anthers containing tetrads of microspores were fixed for 2 h in 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4 (Terasaka et al. 1986), and rinsed several times in buffer. Following dissection of anthers, tetrads were stained with 2.5 μg/ml 3,3′-dihexyloxacarbocyanine ($DiOC_6$) at 4°C, rinsed with buffer, mounted in moviol, and examined by laser scanning confocal microscopy.

For scanning electron microscopy mature pollen was acetolyzed in acetic anhydride and concentrated sulfuric acid (9:1 v/v) in an icewater bath. Pollen was then washed, placed on stubs, air-dried, sputter-coated with gold and examined in an ISI-DS 130 scanning electron microscope. Transmission electron microscopy was performed according to standard procedures (Pérez-Muñoz et al. 1993a,b).

Results and discussion

Mature pollen grains of *Vigna vexillata* are triangular to rounded in polar view and oblong (60×75 μm) in equatorial view. Grains are triporate with opercula composed of globules covering each aperture (Figs. 1, 2). Ektexine ornamentations comprise a coarse reticulum of solid muri surrounding polygonal lumina. The structure of the ektexine is granular (Fig. 3). The ektexine overlies a homogeneous-to-lamellar, electron-dense endexine, which surrounds a thick, microfibrillar intine (Pérez-Muñoz et al. 1993a).

Early development of pollen exine ornamentations occurred on microspores enclosed as a tetrad by the thick callosic jacket formed during meiotic prophase. During exine pattern formation the microtubule cytoskeleton underwent a number of organizational and conformational changes, some of which were congruent with exine ornamentation.

In young unpatterned microspores within the tetrad, microtubules were oriented in concentric rays around the surface of the nucleus. From the nuclear surface, microtubules radiated to the cortex of the cell (Fig. 4). This radial fibrillar cytoskeleton was not visible in the outermost cortical cytoplasm, where fluorescent-labeled anti-tubulin gave a more diffuse fluorescent signal (Fig. 4).

With further development, the difference in cytoskeletal organization between inner and outer cytoplasm became more evident. The intensity of the diffuse cortical fluorescence signal increased coincident with protrusion of apertural sites and patterning of primexine (Figs. 5, 6). The radial microtubule system of the inner cytoplasm persisted until the end of the tetrad stage (Figs. 5, 8). Diffuse cortical fluorescence was eventually replaced by microtubules or bundles of microtubules arranged in clusters that coincided in their placement with apertural sites and with sites subsequently occupied by the lumina of the reticulate wall (Figs. 7–9). The appearance of this patchy system of microtubules occurred at the same time as the onset of patterned wall formation (Figs. 7, 9).

By the time free microspores were released from the callose wall of the tetrad, reticulate exine sculpturings had formed, and an inner exine layer (endexine) had also developed and was highly autofluorescent (Fig. 10). Microtubules in the cytoplasm were again predominantly radially oriented, and the patchy cortical cytoskeleton typical of the tetrad stage (Fig. 7) was no longer present (Fig. 10).

Staining of endomembranes with $DiOC_6$ yielded a pattern similar to that observed using anti-tubulin antibodies. Pre-pattern microspores showed fluorescent $DiOC_6$ signal throughout the cytoplasm (Figs. 11, 12). At the onset of exine formation $DiOC_6$ continued to label endomembranes throughout the cytoplasm (Fig. 13). In addition, a patchy signal appeared in the cortical cytoplasm with fluorescent patches coinciding in position with apertural and luminar sites (Figs. 13, 14). Older microspores in which the patterned autofluorescent primexine was evident did not exhibit patterned cortical $DiOC_6$ labeling, but showed granular cytoplasmic labeling (Fig. 15), similar to that seen in young pre-pattern tetrad members (Figs. 11, 12).

These results are intriguing because they showed for the first time congruence between the pattern of the mature exine, the organization of the microtubule cytoskeleton, and the arrangement of endomembranes. The observed correlation between cortical microtubules and endomembranes labeled by $DiOC_6$ is in accord with numerous reports of interdependency between these two subcellular structures (see, for example, Terasaki et al. 1986) and is suggestive of interaction of microtubules and endomembranes during the development of the patterned microspore wall. Experimental studies involving treatment of developing microspores of *Lilium* and *Tradescantia* with microtubule inhibitors indicate that microtubules do not play a direct role in wall patterning in these species (Sheldon and Dickinson 1986; Tiwari and Gunning 1986; Owens et al. 1990). However, the spatial and temporal correlations between microtubule and exine patterns observed in *Vigna vexillata* grains are strongly suggestive of microtubule involvement in pollen wall pattern determination and/or pattern development in this taxon. Thus, in light of these findings, the hypothesis of microtubule participation in pollen wall pattern formation and development deserves further investigation.

Acknowledgements This study represents a portion of a dissertation submitted by the first author in partial fulfillment of the requirements for the PhD degree at the University of California, Davis. The authors thank Drs. Suresh Tiwari and Nancy Smith-Huerta for advice and assistance in the early stages of this work, Dr. Dallas Hyde and Mr. David Magliano for access to and assistance with confocal microscopy, and Dr. Darlene Southworth for helpful comments on an earlier draft of this manuscript. This research was supported in part by the University of Puerto Rico and by the Research Mentorship Program of the University of California.

References

Blackmore S, Barnes SH (1990) Pollen wall development in angiosperms. In: Blackmore S, Barnes SH (eds) Microspores: evolution and ontogeny. Academic Press, London, pp 173–192

Dickinson HG (1970) Ultrastructural aspects of primexine formation in the microspore tetrad of *Lilium longiflorum*. Cytobiologie 1:437–449

Dickinson HG, Sheldon JM (1984) A radial system of microtubules extending between the nuclear envelope and the plasma membrane during early male haplophase in flowering plants. Planta 161:86–90

Heslop-Harrison J (1971) Wall pattern formation in angiosperm microsporogenesis. Symp Soc Exp Biol 25:277–300

Owens SJ, Sheldon JM, Dickinson HG (1990) The microtubular cytoskeleton during pollen development. Plant Syst Evol [Suppl. 5]:31–37

Pérez-Muñoz CA, Jernstedt JA, Webster BD (1993a) Pollen wall development in *Vigna vexillata*. I. Characterization of wall layers. Am J Bot 80:1183–1192

Pérez-Muñoz CA, Jernstedt JA, Webster BD (1993b) Pollen wall development in *Vigna vexillata*. II. Ultrastructural studies. Am J Bot 80:1193–1202

Rowley JR, Skvarla JJ (1987) Ontogeny of pollen in *Poinciana* (Leguminoseae). II. Microspore and pollen grain periods. Rev Palaeobot Palynol 50:313–331

Sheldon JM, Dickinson HG (1983) Determination of patterning in the pollen wall of *Lilium henryi*. J Cell Sci 63:191–208

Sheldon JM, Dickinson HG (1986) Pollen wall formation in *Lilium*: the effect of chaotropic agents, and the organisation of the microtubular cytoskeleton during pattern development. Planta 168:11–23

Skvarla JJ, Rowley JR (1987) Ontogeny of pollen in *Poinciana* (Leguminoseae). I. Development of exine template. Rev Palaeobot Palynol 50:293–311

Smith-Huerta NL, Jernstedt JA (1989) Root contraction in hyacinth. III. Orientation of cortical microtubules visualized by immunofluorescence microscopy. Protoplasma 151:1–10

Takahashi M (1989) Pattern determination of the exine in *Caesalpinia japonica* (Leguminosae: Caesalpinioideae). Am J Bot 76:1615–1626

Terasaki M, Chen LB, Fujiwara K (1986) Microtubules and the endoplasmic reticulum are highly interdependent structures. J Cell Biol 103:1557–1568

Tiwari S (1989) Cytoskeleton during pollen development in *Tradescantia virginiana*: a study employing chemical fixation, freeze-substitution, immunofluorescence, and colchicine administration. Can J Bot 67:1244–1253

Tiwari S, Polito VS (1990) The initiation and organization of microtubules in germinating pear (*Pyrus communis* L.) pollen. Eur J Cell Biol 53:384–389

van Uffelen GA (1991) The control of spore wall formation. In: Blackmore S, Barnes SH (eds) Pollen and spores: patterns of diversification. Clarendon Press, Oxford, pp 89–102

Wick SM, Muto S, Duniec J (1985) Double immunofluorescence labeling of calmodulin and tubulin in dividing plant cells. Protoplasma 126:198–206