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The microtubular cytoskeleton during development of the zygote, proembryo and free-nuclear endosperm in *Arabidopsis thaliana* (L.) Heynh

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Abstract. The microtubular cytoskeleton has been studied during development of the zygote, proembryo and free-nuclear endosperm in *A. thaliana* using immunofluorescence localization of tubulin in enzymatically isolated material. Abundant microtubules (MTs) are found throughout proembryogenesis. Microtubules in the coenocytic endosperm are mainly internal. By contrast, there is a re-orientation of MTs to a transverse cortical distribution during zygote development, predominantly in a subapical band which accompanies a phase of apical extension. The presence of these cortical arrays coincides with the elongation of the zygote. Cortical arrays also accompany elongation of the cylindrical suspensor. Extensive networks of MTs ramify throughout the cytoplasm of cells in the proembryo proper. Perinuclear arrays are detected in a number of cell types and MTs contribute to typical mitotic configurations during nuclear divisions. Preprophase bands of MTs are absent throughout megasporogenesis and embryo-sac development and do not occur in endosperm cell divisions. We have observed MTs throughout the first division cycle of the zygote. By placing the observed stages in a most probable sequence, we have identified this cell cycle as the point during embryogenesis at which a preprophase band is reinstated as a regular feature of cell division. Preprophase bands were observed to predict planes of cytokinesis in cell divisions up to the octant stage.

Key words: *Arabidopsis* (Cytoskeleton in proembryogenesis) – Cytoskeleton – Endosperm (free-nuclear) – Microtubule – Preprophase band – Proembryo – Zygote

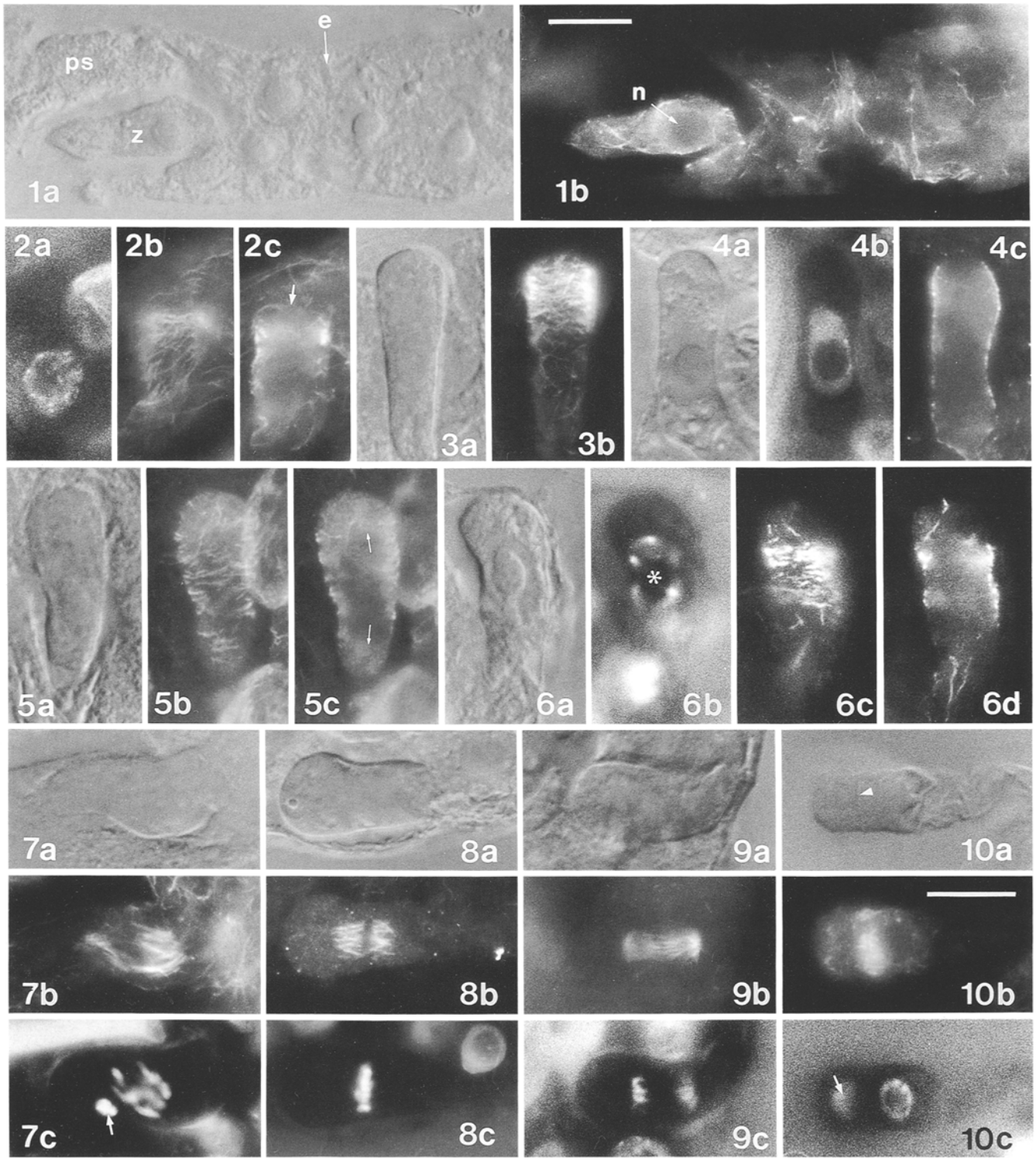
Introduction

Many of the morphological, cellular and biochemical aspects of embryogenesis in flowering plants have been well studied (for review, see Raghavan 1986). Despite the undoubted importance of the microtubular cytoskeleton throughout the processes which lead to formation of the embryo and endosperm, studies which refer to microtubules (MTs) during any phase of embryogenesis are scarce (Singh and Mogensen 1975; Van Lammeren 1988a; Huang et al. 1990). *Arabidopsis thaliana* (L.) Heynh. is widely used in experimental plant biology owing to its many favourable features, such as a small genome and short generation time. Embryo development in *A. thaliana* largely follows the *Capsella* variation of the Onagrad type (Misra 1962; classification system of Johansen 1950). The basic cytology of events is well established (e.g. Misra 1962; Yakovlev and Alimova 1976) and embryo-lethal mutants have been isolated which provide valuable insight into developmental regulation of the embryo (Meinke and Sussex 1979; Marsden and Meinke 1985). In this study we follow the changing distributions of MTs through early post-fertilization development in *A. thaliana*, up to the octant (eight-celled) proembryo stage. Extensive MT arrays occur in all cell types. Varying patterns of distribution of MTs appear to reflect a series of disparate roles within the different cells and stages of proembryogenesis. We used indirect immunofluorescence microscopy to localize MTs in enzymatically isolated embryo sacs and proembryos.

A preprophase band of MTs (PPB) is a marker of the site where new cell walls will be positioned at cytokinesis (see Gunning and Hardham 1982). It is well known to be present in the meristems and meristemoids during vegetative growth, but its development is suppressed during megasporogenesis and subsequent embryo-sac development (Hogan 1987; Webb and Gunning 1990a, b). It has not been known at what stage during embryogenesis the PPB is re-established to become a routine part of spatial regulation of cell division. In the present study we propose that it is reinstated as early as the first zygotic division and that it is present consistently in somatic divisions thereafter during proembryogenesis.

Preliminary results of part of this study have been published previously (Webb and Gunning 1990b).

Abbreviations: DIC = differential interference contrast optics; MT = microtubule; PPB = preprophase band of microtubules



Figs. 1–10. The zygote of *Arabidopsis thaliana*. Isolated and immunofluorescence-labelled cells. Scale bars = 10 μm

Fig. 1. a Fertilized embryo sac showing the young zygote (*z*), the degenerating persistent synergid (*ps*) and the free-nuclear endosperm (*e*) (DIC). **b** Microtubules are found throughout the cytoplasm of the endosperm and zygote in random orientation and are particularly concentrated around the zygote nucleus (*n*). Note the lack of fluorescence in the synergid. X1420

Figs. 2–6. Micropylar end of the zygote is at the bottom of each micrograph. X1510

Fig. 2. a The young zygote in which the nucleus is spherical. Cortical (**b**) and (**c**) mid-plane views show a band of MTs transversely encircling the cell at the distal end (with respect to the micropyle). The orientation of cortical MTs gradually shifts to approaching longitudinal at the proximal end. *Arrow* = distal boundary of the zygote

Fig. 3. a Elongating zygote (DIC). **b** Microtubules are predominantly cortical and transversely oriented with a distinct concentration at the growing end of the cell

Material and methods

Plants of *Arabidopsis thaliana* (L.) Heynh. var. *Columbia* (kindly provided by T. Caspar, Michigan State University, E. Lansing, USA) were grown under constant illumination at 18° C. A starchless mutant (mutant line TC7; Caspar et al. 1985) was used because its cells are optically clearer than those with starch grains. Fully opened flowers with pistils of 3–3.5 mm were found to contain ovules at the appropriate stages of zygote and proembryo development.

Fertilized embryo sacs and proembryos were enzymatically isolated and processed for indirect immunofluorescence localization of MTs as described in Webb and Gunning (1990a). Briefly, pistils were fixed in 4% paraformaldehyde before the ovules were dissected out of the ovary, digested in an enzyme solution containing 2% cellulase and 2% pectinase, and the cells separated by gentle squashing. Cells were further made permeable using 10% dimethyl sulphoxide and 3% Nonidet NP–40, incubated with anti-α-tubulin antibody and subsequently with fluorescein isothiocyanate conjugated to a sheep anti-mouse immunoglobulin-G antibody. Finally, preparations were treated with a Hoechst stain for DNA localization and examined using a Nikon Optiphot microscope equipped with differential interference contrast (DIC) optics and a 100–W high-pressure mercury lamp, epifluorescence optics and appropriate filters. Photomicrographs were made with Kodak T–MAX 400 film (ASA 400 or 1600) and T–MAX developer (both Eastman-Kodak, Rochester, N.Y., USA).

Results

Our observations on the anatomical events of early embryogenesis in *Arabidopsis thaliana* agree with previous descriptions (e.g. Misra 1962; Yakevlov and Alimova 1976). Hence in this report we give limited anatomical information, and only where necessary for a full appreciation of the cytoskeletal distributions which we report. The events described occur within a period that lasts approximately 16 h, using the time scale of Mansfield and Briarty (1987).

The zygote. Following fertilization in *A. thaliana,* the club-shaped zygote is located at the micropylar end of the embryo sac and is surrounded by the developing free-nuclear endosperm (Fig. 1). The microtubular cytoskeleton was visualized by immunofluorescent labelling of its α-tubulin component. We examined more than 200 zygotes in an effort to observe all stages in the development of MT arrays during the first cycle of growth and division in the new sporophyte generation. The changes in MT arrangement could be placed in a sequence by reference to cell length. Figure 11 divides them arbitrarily into ten stages. We calculated the relative frequency with which each stage occurs as a percentage of the total number of zygotes observed (Fig. 11).

In the newly formed zygote, MTs are found throughout the cytoplasm in random orientation and are particularly concentrated in the perinuclear region (Figs. 1, 11.1). Subsequently, MTs are found primarily in the cell cortex. In the young zygote a distinct band of cortical MTs develops, oriented transversely with respect to the future proembryo-suspensor axis (Figs. 2, 11.2). The band is formed at the distal end of the cell with respect to the micropyle and coincides with marked elongation of the zygote, we suspect mainly in the sub-apical region, perpendicular to the orientation of the cortical MTs located there. Along the length of the zygote there is a gradual shift in MT orientation so that at the proximal end MTs are mainly longitudinally oriented (Figs. 2, 11.2).

As the zygote elongates, transverse cortical MTs become the dominant feature of the microtubular cytoskeleton along most of its length (Figs. 3–5). A reduced number of MTs persist through the internal cytoplasm, except close to the proximal end of the cell where they are found throughout the cytoplasm in approximately longitudinal orientation until mitosis (e.g. Fig. 6d). While the zygote is elongating, the band of MTs toward the distal end remains distinct (Figs. 3, 11.3), then becomes less prominent as the cell reaches its maximum length (Figs. 4, 11.4). At maturity the MTs are fairly evenly spaced along the cell (Figs. 5, 11.5). The zygote remains in Stages 2–5 (Fig. 11) for relatively long periods, as indicated by the high frequencies with which they occur (Fig. 11). The nucleus is approximately spherical in the young zygote (Fig. 2a), but it elongates while the cell extends apically (Figs. 4b, 5c). The elongated state persists as the DNA begins to condense before division (Fig. 6b).

Fig. 4. a Zygote which has almost reached its maximum length (DIC). **b** The nucleus is elongating into the newly formed portion of the cell. **c** Mid-plane view of the cell. Microtubules are largely confined to the cortex in transverse orientation. The concentration toward the tip is less pronounced than in Fig. 3

Fig. 5. a Mature zygote (DIC). **b, c** Surface (**b**) and mid-plane (**c**) views showing an even distribution of cortical MTs in a net transverse orientation. *Arrows* indicate the extremities of the fully elongated nucleus

Fig. 6. a Zygote entering preprophase (DIC). **b** The nuclear DNA is condensing (* = nucleolus which is still present). **c, d** Cortical (**c**) and mid-plane (**d**) views of a broad PPB which girdles the cell at the putative site of cell-plate formation. A number of longitudinal MTs are still present at the proximal end of the zygote

Figs. 7–10. Micropylar end of the zygote is at the right of each micrograph. X1510

Fig. 7. a Zygote during late prophase of mitosis (DIC). **b** Microtubules are confined to a spindle array. **c** Hoechst staining shows the chromosomes to be fully condensed. *Arrow* = disintegrating DNA in the synergid which is overlapping with the zygote in this preparation

Fig. 8. a Zygote during metaphase (DIC). **b** Virtually all discernable MTs contribute to the metaphase spindle. **c** The chromosomes lie along the equator of the spindle

Fig. 9a–c. Zygote during anaphase of mitosis, showing (**a**) the cell boundary (DIC), (**b**) the MTs contributing to the anaphase spindle array, and (**c**) the chromosomes moving towards the spindle poles

Fig. 10a–c. Zygote during telophase. **a** Differential Interference Contrast image of the cell showing the newly-forming cell plate *(arrowhead)*. **b** A phragmoplast array of MTs is present in the interzone between daughter nuclei. A second population of MTs is closely associated with the nuclei. **c** Hoechst staining of DNA shows the daughter nuclei. One nucleus *(arrow)* is out of focus in this plane

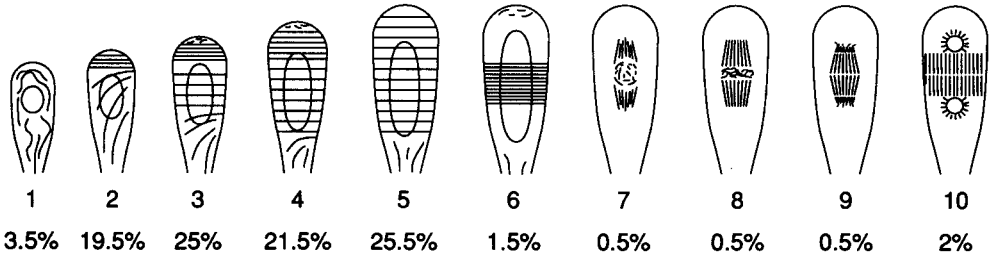
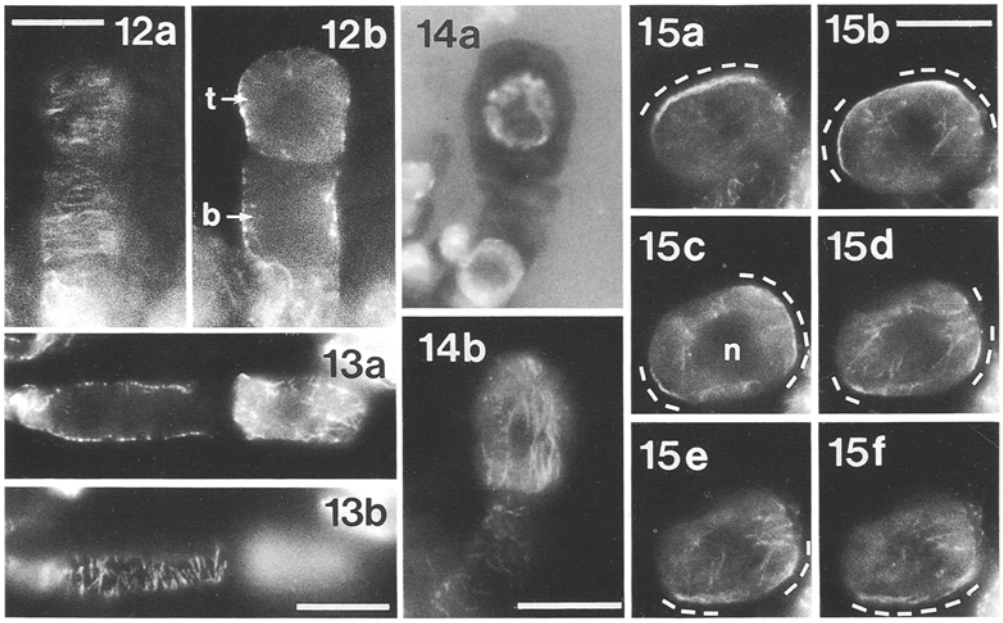


Fig. 11. Summary of the changing MT distributions during zygote development in *A. thaliana*. The stages numbered *1–10* correspond to those shown in Figs. 1–10. The frequency with which each stage was observed is indicated as a percentage of the total 200 zygotes examined



Figs. 12–15. Immunofluorescently labelled, isolated cells at the stage directly following division of the zygote in *A. thaliana*. Scale bars = 10 μm. **Fig. 12a, b.** Surface (**a**) and (**b**) median planes of focus showing cortical arrays of transversely oriented MTs in both the terminal (*t*) and basal (*b*) cells of the proembryo. A few randomly oriented MTs are also detected through the internal cytoplasm of the terminal cell. X1230. **Fig. 13. a** Later stage than Fig. 12, showing that the MTs in the terminal cell are reoriented to form a complex network ramifying throughout the cytoplasm, while MTs are confined to the cortex in the basal cell. **b** Surface view of the basal cell showing the cortical MTs in transverse orientation. X1220. **Fig. 14.** The terminal cell of *A. thaliana* just before the first longitudinal division. **a** The nucleus is beginning to condense. **b** A wide PPB longitudinally encircles the cell. Note the basal cell is still in interphase. X1310. **Fig. 15a–f.** The terminal cell just before division. (*Note:* it has become detached from the basal cell during processing.) Panels **a–f** show successive focal planes, collectively displaying a PPB girdling the cell. A number of MTs are still detected in the internal cytoplasm. *n* = Nucleus; --- = parts of PPB visible in focal plane. X1290

Prior to mitosis, a broad putative PPB develops in the elongated zygote, at the position of the nucleus and distinctly more basal than the preceding sub-apical girdle. This is known to correspond to the position of the future cell plate (Figs. 6, 11.6). A narrow, more advanced PPB was not observed, probably reflecting the extremely short duration of this stage (Gunning and Sammut 1990). As mitosis progresses, typical spindle configurations of MTs are exhibited through prophase (Figs. 7, 11.7), metaphase (Figs. 8, 11.8) and anaphase (Figs. 9, 11.9), with few MTs detectable through the rest of the cytoplasm. By late telophase a phragmoplast array of MTs between daughter nuclei signifies cell-plate formation (Figs. 10, 11.10). A second population of MTs is closely associated with the nucleus during this stage (Fig. 10). The very low frequencies of mitotic figures observed (Fig. 11) indicate that the division processes are very rapid.

Proembryo development. The enzymatic isolation technique we employed allowed the majority of proembryos to be isolated intact, indicating a tight coherence of their constituent cells. The asymmetrical first division of the zygote produces a small terminal cell, which is the precursor of the embryo proper, and a larger basal cell which divides subsequently to form the suspensor.

The proembryo proper. Initially two main populations of MTs occur in the terminal cell (Fig. 12): (i) a cortical array oriented perpendicular to the direction of cell el-

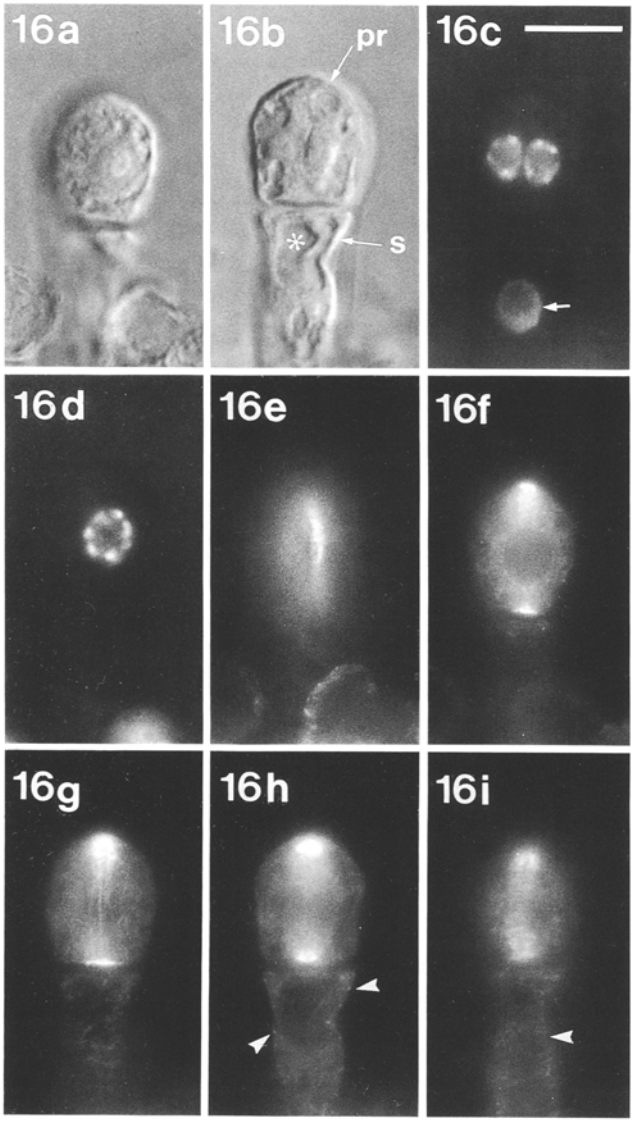


Fig. 16a–i. Immunofluorescence-labelled, isolated proembryo of *A. thaliana* in the process of dividing from the two-celled to quadrant (four-celled) proembryo-proper stage. Scale bar = 10 μm; X1230. **a** Upper plane of focus showing the "front" cell of the proembryo proper (DIC). **b** Lower plane showing the "back" cells of the proembryo proper (*pr*) and the developing suspensor (*s*) which is somewhat vacuolate (* = vacuole). **c, d** Hoechst staining of the nuclei, corresponding to the focal planes of **b** and **a**, respectively. **c** The cell of the proembryo proper has divided to form two daughter nuclei. The suspensor nucleus *(arrow)* is also visible. **d** The nucleus of the front cell is in preprophase, with the chromosomes beginning to condense. **e–i** Microtubules in five planes of focus (focussing down through the cells). **e** Cortical view of the PPB which encircles the "front" cell longitudinally. **f** Mid-plane view of the "front" cell. **g** Plane of focus which encompasses the cortex of the "front" cell in which the PPB is visible, and directly in line with this PPB, a phragmoplast in the tightly appressed "back" cell. **h** Mid-plane view of the "back" cell. **i** Cortical view of the phragmoplast in the "back" cell which signifies cell-plate formation between the nuclei; MTs in the suspensor are largely confined to the cell cortex *(arrowheads)*

ongation, and (ii) a lesser number which occurs in random orientation through the rest of the cytoplasm. When elongation of the cell ceases the MT system becomes a complex reticulate array which ramifies throughout the cytoplasm (Fig. 13). An array of parallel cortical MTs is no longer observed.

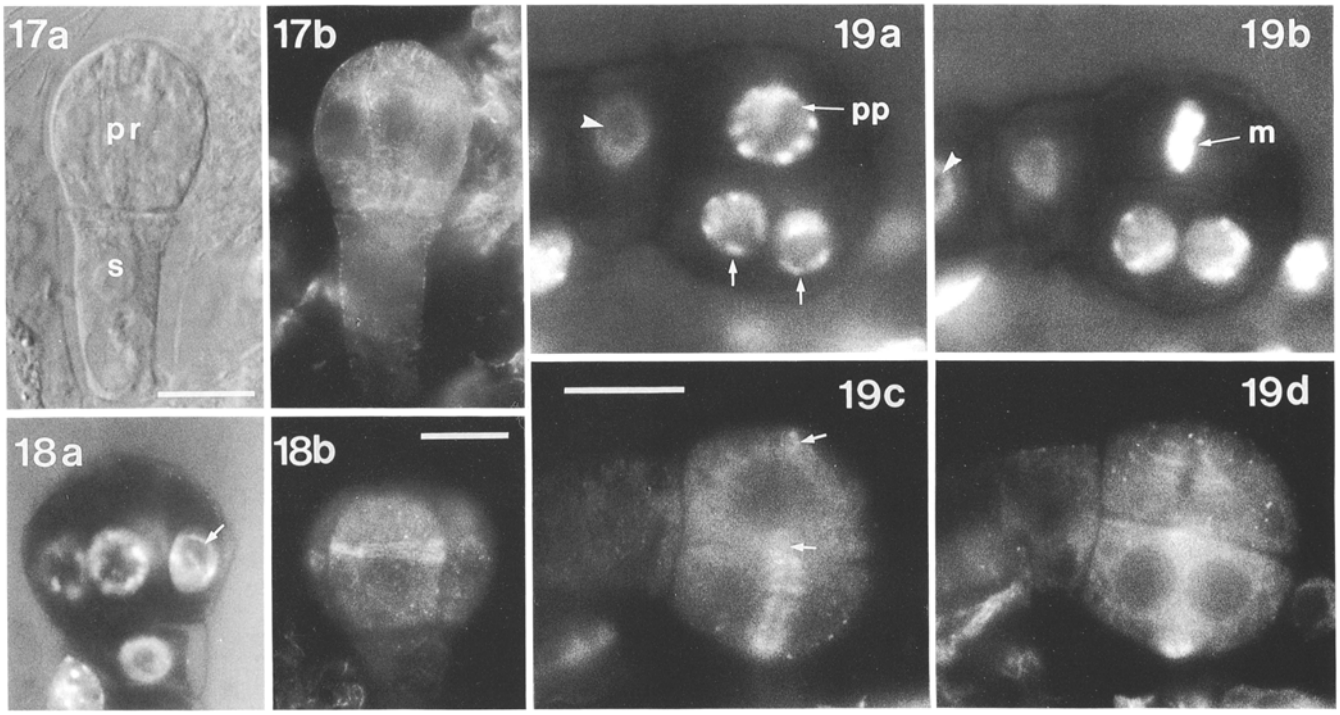
Development of a longitudinal PPB girdling the terminal cell precedes the first, longitudinal, division of the proembryo (Figs. 14, 15). A broad band first appears (Fig. 14) which progressively becomes narrower (Fig. 15), thus resembling the pattern of PPB development in other meristematic plant cells (Wick and Duniec 1983; Gunning and Sammut 1990). Internal MTs remain detectable during this phase (Fig. 15). Longitudinal divisions then occur in both products of the first division, at right angles to the first (Fig. 16), to produce a four-celled proembryo (quadrant stage). A fine PPB heralds the onset of mitosis (Fig. 16e–g) and cell-plate formation is indicated by a phragmoplast array of MTs which forms in the plane predicted by the PPB in the interzone between daughter nuclei (Fig. 16g–i). Cell-plate growth is centrifugal, as indicated by persistence of MTs at the margins of the newly forming cell plate.

Microtubules in the quadrant proembryo are plentiful and found throughout the cytoplasm in random orientation (Fig. 17). Parallel cortical arrays were never observed. Preprophase bands also precede the next round of mitotic divisions, which occur transversely in the quadrant cells (Fig. 18). The characteristic appearance of nuclei in preprophase-prophase (e.g. Figs. 16, 18) verified that cells in which we identified putative PPBs were in the appropriate stage of the cell cycle. A high proportion of PPBs in the proembryos examined probably reflects a relatively long duration of preprophase in these cells. The divisions which produce the quadrant and octant (eight-celled) proembryos are not strictly synchronous and hence MT configurations characteristic of different division stages may be observed simultaneously in a single proembryo (Figs. 16, 19).

The octant proembryo has a complex network of MTs which extends throughout the cytoplasm of each cell (Figs. 20, 21). In the cells of the young octant the MTs are closely associated with the nuclei and are particularly concentrated in those areas which face other member cells (Fig. 20). As the octant stage matures, the randomly arranged MTs in the cortex of each cell (Fig. 20) become partly re-oriented into a parallel array (Fig. 21). Internal MTs remain abundant (Fig. 21). High background fluorescence is observed throughout development of the proembryo proper, possibly indicating the presence of much free tubulin.

The suspensor. Concurrently with development of the proembryo, the basal cell undergoes a series of transverse divisions to produce a uniseriate suspensor. These divisions are preceded by PPBs (not shown). The basal cell and each subsequent cell of the developing suspensor has an interphase array of parallel cortical MTs, oriented transversely to the direction of elongation of the suspensor. Few MTs are discernible in the internal cytoplasm (e.g. Figs. 13, 17). The suspensor cells often become highly vacuolated (e.g. Fig. 16).

The persistent synergid and the free nuclear endosperm. Shortly after fertilization the persistent synergid de-



Figs. 17–19. Immunofluorescence labelled, isolated proembryos of *A. thaliana* during the quadrant stage. Scale bars = 10 μm

Fig. 17. a Cells during interphase showing the proembryo proper (*pr*) and the suspensor (*s*) (DIC). **b** Microtubules are found in random orientation throughout the cytoplasm of the proembryo proper and mainly confined to transverse cortical arrays in the suspensor. X1230

Fig. 18a, b. Proembryo-proper cells at preprophase. X1190. **a** Hoechst staining showing the nuclei, in which the chromosomes are starting to condense, except one *(arrow)* which is lagging behind. **b** Focussing at the surface of the front cell to show the PPB which encircles the cell transversely, predicting the future division site

Fig. 19a–d. Two planes of focus showing division of the quadrant to form the octant (eight-celled) proembryo proper. X1600. **a, b** Hoechst staining of the nuclei. **a** Upper focal plane showing one nucleus in preprophase-prophase (*pp*) and two smaller nuclei which are the products of recent division *(arrows)*. **b** Lower plane showing two further daughter nuclei situated in the cell behind those shown in **a** and chromosomes at metaphase (*m*) situated in the cell behind the preprophase-prophase cell of **a.** Suspensor nuclei are also visible *(arrowheads)*. **c, d** Microtubules corresponding to the focal planes of **a** and **b,** respectively. **c** A PPB *(arrows)* transversely girdles the cell in preprophase-prophase and a parallel phragmoplast array has formed between daughter nuclei in the adjacent cell. **d** A spindle array is visible in the metaphase cell and a late-stage phragmoplast array in the adjacent cell, with MTs persisting at the growing margins of the cell plate

generates. This is accompanied by a loss of those MTs detectable by immunofluorescence microscopy (Figs. 1, 22).

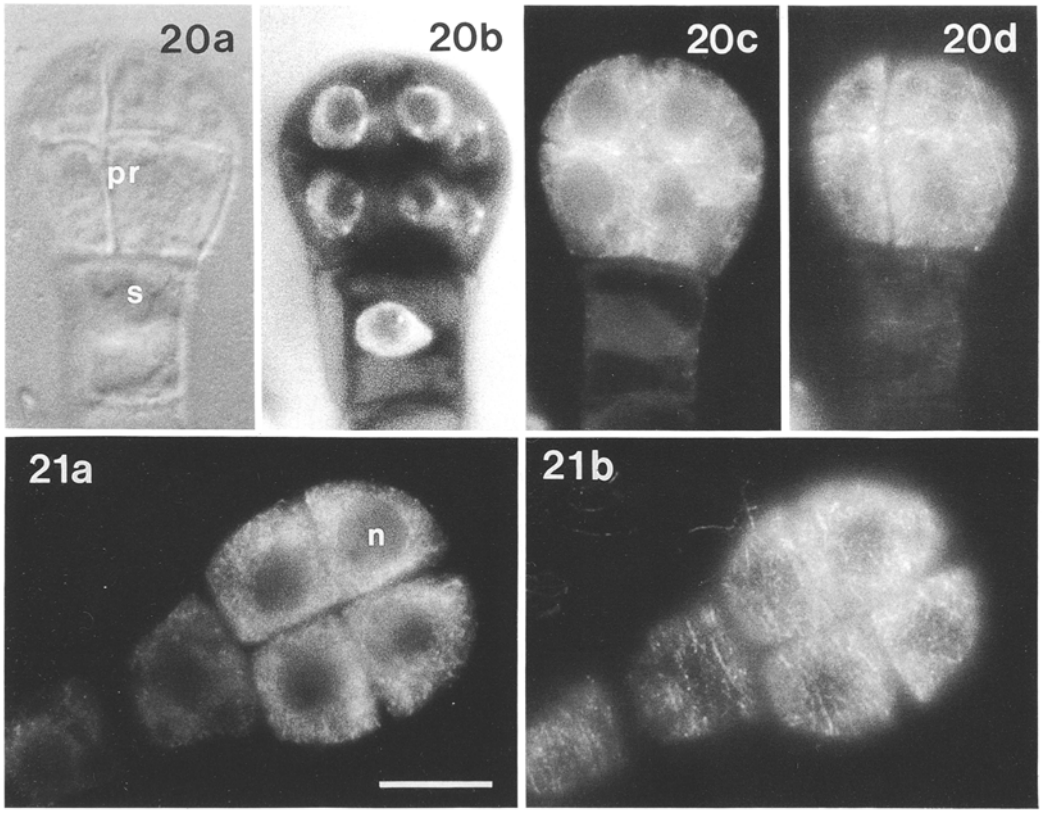
The primary endosperm nucleus of *A. thaliana* divides almost immediately after its formation, and divisions continue without wall formation throughout the period examined in this study (i.e. to the octant proembryo stage). The endosperm has a large central vacuole (Fig. 22). During interphase MTs are found throughout the cytoplasm in random orientation (Fig. 1). Microtubules also radiate from each interphase nucleus (Fig. 22).

No PPBs are observed in the coenocytic endosperm. As the chromosomes condense during early prophase, MTs become strongly localized around the nuclei (Fig. 23) and subsequently contribute to the spindle configurations (Fig. 24). Phragmoplast-type arrays of MTs occur between nuclei during telophase (Fig. 24) and later disperse unaccompanied by cell-plate formation. During nuclear divisions few MTs are found outside the spindle arrays (Figs. 23, 24). Mitoses in the endosperm nuclei approach synchrony but different stages may be found in a given preparation. Many MTs extend between daughter nuclei after divisions (Fig. 25) and the interphase network is soon re-established.

Discussion

Our observations on the changing organization of the cytoskeleton contribute new insights into embryogenetic processes in flowering plants. In the present study we found that extensive arrays of MTs occur in all cells involved in proembryogenesis in *Arabidopsis thaliana*. These arrays can be placed into five distinct classes.

The *first category* comprises MTs in the cell cortex. Interphase cortical arrays become apparent during zygote maturation, and are present subsequently throughout proembryogenesis. Our results augment the report of MTs in the parietal cytoplasm of the 6–12-celled proembryo in *Quercus* (Singh and Mogensen 1975). Before and after the initial division of the zygote, and throughout all



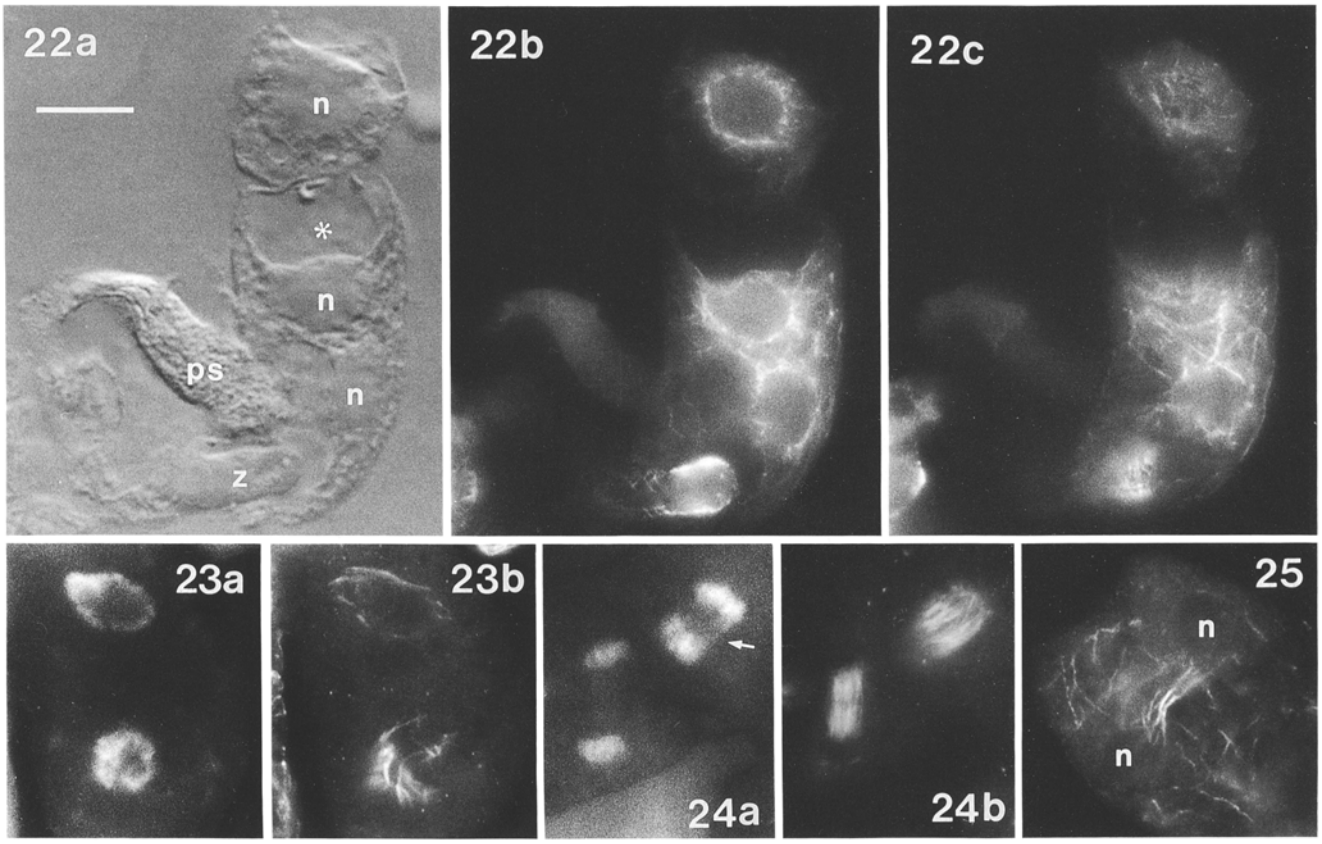
Figs. 20, 21. Immunofluorescently labelled, isolated proembryos of *A. thaliana* during the octant stage. Scale bar = 10 μm; X1520. **Fig. 20a–d.** Young octant. **a** Surface view showing four cells of the proembryo proper (*pr*) attached to the uniseriate suspensor (*s*). **b** Slightly lower focal plane than **a** showing corresponding nuclei after Hoechst staining. **c, d** Microtubules in two focal planes corresponding to **b** and **a**, respectively. Microtubules are abundant throughout the cytoplasm of the proembryo-proper cells. **Fig. 21.** Mature octant shown in two planes of focus. **a** Median plane through four cells of the proembryo proper showing MTs ramifying throughout the internal cytoplasm (*n* = position of nucleus in one cell). **b** Surface view showing transverse cortical MT arrays forming in the proembryo proper cells. The MTs are largely confined to such cortical arrays in the suspensor cells

stages of suspensor growth, these MTs are arranged perpendicular to the direction of cell elongation. Such configurations are typical of somatic cells during interphase and they are generally accepted as having a major role in cell-shaping (see Gunning and Hardham 1982). Elongation of the zygote, probably by subapical growth, coincides with the occurrence of a transverse girdle of MTs near the apex of the cell. Similar MT arrays have been reported in *Adiantum* protonemata (Murata et al. 1987) and *Azolla* root hairs (Cleary 1989) in which growth activity is also largely restricted to the apical region of the cell.

Re-orientation of MTs to a random arrangement in the terminal cell coincides with an observed change in cell growth from elongation to radial expansion. Similarly, cortical MTs are oriented randomly in ensuing cells of the embryo proper, in which enlargement is isotropic during the next two rounds of division. There is a transition toward a transverse cortical array as the octant-stage proembryo matures. The lack of an array of MTs at the surface of the coenocytic endosperm indicates that its shape is influenced more by the surrounding nucellus rather than being internally determined. This phenomenon is apparent throughout the development of the female reproductive cells in *A. thaliana* (Webb and Gunning 1990a, b) and in other angiosperm species such as *Gasteria verrucosa* (e.g. Willemse and Van Lammeren 1988). By contrast, the proembryo has no tissue immediately around it with sufficient mechanical rigidity to provide for such external control of its shape. The appearance of cortical MTs in the zygote accords with the idea that endogenous shape control, lost at megasporogenesis and absent throughout megagametogenesis, is one of the first morphogenetic control systems to be restored in the new sporophytic generation. Assumption of an axis of polarity, correlated with MT distributions, clearly precedes the first round of mitosis.

The *second type* of MT population occurs in the internal cytoplasm during interphase. Internal MTs are detected throughout the cytoplasm of the free-nuclear endosperm and zygote, and are particularly abundant in all cells of the proembryo proper. Our observations accord with the recent description of this category of MTs in globular-stage wheat embryos (Van Lammeren 1988a). These MTs may help to maintain cytoplasmic organization. A loss of fluorescence from internal MTs in the persistent synergid as it degenerates is similarly found in other degenerating cell types in the young ovule (e.g. Webb and Gunning 1990a).

The *third* distinct category of MTs we observed was found in the perinuclear region. The occurence of extensive networks of MTs radiating from the interphase nuclei in the free-nuclear endosperm closely resembles the patterns found in the same stages of endosperm development in wheat (Van Lammeren 1988b) and in many other multinucleate cells (e.g. Woodcock 1971; LaClaire 1987). Such MTs are believed to contribute to the correct spacing of the nuclei in the coenocytic phase (Van Lammeren 1988b). Similarly, we observed large concentrations of MTs closely associated with the nuclei in the young zygote and octant proembryo. They too may also have a role in nuclear positioning.



Figs. 22–25. Immunofluorescence labelled, isolated free-nuclear endosperm of *A. thaliana*. Scale bar = 10 μm; X1300

Fig. 22a–c. Fertilized embryo sac of *A. thaliana*. **c** Differential interference contrast image showing the endosperm, zygote (*z*), and degenerating persistent synergid (*ps*) which has been somewhat displaced during processing. Three nuclei (*n*) are visible in the vacuolate endosperm (* = vacuole). **b, c** Two focal planes showing MTs throughout the endosperm cytoplasm in random orientation with an extensive network radiating from each nucleus. Note also the transverse cortical array in the zygote and the absence of MT fluorescence in the synergid

Fig. 23a, b. Endosperm nuclei during prophase. **a** Hoechst staining showing the condensing nuclei. **b** Microtubules are localized around each nucleus

Fig. 24a, b. Portion of the endosperm during mitoses of two nuclei showing (**a**) the positions of chromosomes after Hoechst staining and (**b**) the corresponding configurations of MTs. One nucleus is in anaphase *(arrow)* while the other is in late telophase with a phragmoplast-type array of MTs between groups of chromosomes. Few MTs are found in the rest of the cytoplasm during mitoses

Fig. 25. Portion of the endosperm shortly after nuclear division, showing bundles of MTs extending between daughter nuclei (*n*). Microtubules ramify throughout the rest of the cytoplasm

Spindle and phragmoplast MTs constitute the *fourth class* of MTs in the *A. thaliana* proembryo. In all cells of the post-fertilized embryo sac, MTs contribute to typical spindle configurations during mitoses, including the formation of phragmoplast arrays between daughter nuclei. In cells of the proembryo this is accompanied by centrifugal cell-plate formation; however, in the free nuclear endosperm, where no PPBs precede mitosis, phragmoplast MTs dissipate without cell walls being formed. Similar phragmoplast behaviour has been reported in the free-nuclear endosperm of *Plumbago* (Huang et al. 1990).

The *fifth category* of MTs consists of PPBs. We have sought to establish the point during embryogenesis at which PPBs return as a regular feature of cell division. Along with cortical arrays, they are absent between megasporogenesis and fertilization (Webb and Gunning 1990a, b). A putative PPB was detected as early as the first division of the zygote. The re-establishment of an interphase cortical array of transversely oriented MTs earlier in the same cell cycle would appear to support the suggestion that the ability to form PPBs is dependent on the prior existence of such an array (Doonan et al. 1987). However, the continued presence of PPBs before the subsequent divisions of the proembryo runs counter to this idea, as ordered cortical MT arrays are not detected in these cells.

Our application of immunofluorescence techniques to isolated embryo sacs and proembryos has provided new information on the microtubular cytoskeleton in key developmental stages for which very little information has been obtained by electron microscopy. In future work, use of similar methods should help us in resolving issues concerning the cellularization of the endosperm and cytoskeletal events preceding the development of heterogeneous cell tiers in the embryo.

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