Planta (1989) 179:530–540

Planta

A planar microtubule-organizing zone in guard cells of *Allium*: experimental depolymerization and reassembly of microtubules

J. Marc*, Y. Mineyuki, and B.A. Palevitz**
Department of Botany, University of Georgia, Athens, GA 30602, USA

Abstract. The generation of the unique radial array of microtubules (MTs) in stomatal guard cells raises questions about the location and activities of relevant MT-organizing centers. By using tubulin immunofluorescence microscopy, we studied the pattern of depolymerization and reassembly of MTs in guard cells of *Allium cepa* L. Chilling at 0° C reduces the MTs to small remnants that surround the nuclear surface of cells in the early post-cytokinetic stage, or form a dense layer along the central portion of the ventral wall in older guard cells. A rapid reassembly on rewarming restores either MTs extending from the nuclear surface randomly throughout the cytoplasm in very young cells, or an array of MTs radiating from the dense layer at the ventral wall later in development. A similar pattern of depolymerization and reassembly is achieved by incubation with 100 μM colchicine followed by a brief irradiation with ultraviolet (UV) light. Incubation with 200 μM colchicine leads to a complete depolymerization that leaves only a uniform, diffuse cytoplasmic fluorescence. Nonetheless, UV irradiation of developing guard cells induces the regeneration of a dense layer of MTs at the ventral wall. The layer is again positioned centrally along the wall, even if the nucleus has been displaced by centrifugation in the presence of cytochalasin D. Neither the regenerated layer nor the perinuclear MTs seen earlier are related to the staining pattern of serum 5051, which reportedly binds to centrosomal material in animal and plant cells. The results support the view that, soon after cytokinesis, a planar MT-organizing zone is established in the cortex along the central portion of the ventral wall, which then generates the radial MT array.

Key words: *Allium* – Colchicine – Cytoskeleton – Guard cell – Microtubule – Stomatal complex

Introduction

Microtubule (MT)-organizing centers (MTOCs) are cytoplasmic entities that control the initiation of MTs. Generally they define the number of MTs initiated, their polarity, orientation, length, stability, and temporal and spatial distribution (Pickett-Heaps 1969; Brown et al. 1982; Gunning and Hardham 1982; McIntosh 1983; Tucker 1984; Brinkley 1985; Mazia 1987). Thus MTOCs are of fundamental importance in the establishment, maintenance and dynamics of MT arrays (see also Kirschner and Mitchison 1986).

Various hypotheses have been proposed regarding the organization of interphase MT arrays in higher-plant cells, though the molecular identity of the relevant MTOCs remains unknown. On the basis of electron-microscope studies in the fern *Azolla*, Gunning et al. (1978) suggested that cortical MT arrays are nucleated along cell edges from complexes of electron-dense matrix, short MTs, and vesicles; included in this hypothesis are mechanisms that determine MT orientation and, by selective activation of the complexes, the generation of MT arrays at different subcellular locations. Similar complexes have been reported along the cell edges in guard cells (GCs) in *Zea* (Galatis 1980), *Adiantum* (Galatis et al. 1983) and *Azolla* (Busby and Gunning 1984). On the other hand, although present, the complexes are not restricted to the cell edges in GCs in *Phleum* (Palevitz 1981) or in guard

* To whom correspondence should be addressed
** *Present address:* Botanical Institute, Hiroshima University, Hiroshima, Japan

Abbreviations: GC = guard cell; MT = microtubule; MTOC = microtubule-organizing center; UV = ultraviolet

mother cells of *Zea* (Galatis 1982). As judged by the distribution of MTs recovering after drug-induced depolymerization, MT-nucleating sites appear to be uniformly dispersed throughout the cell cortex in suspension cultures of *Alfalfa* and *Zinnia* (Falconer et al. 1988), or predominantly in the cortex adjacent to the longitudinal cell walls in the root tip of *Zinnia* (Cleary and Hardham 1988). In contrast, according to the binding pattern of the anti-centrosomal serum 5051 in root-tip cells of *Allium*, Clayton et al. (1985) and Wick (1985a, b) suggested that initiation sites for the earliest interphase MTs are located in the perinuclear region, and that a separate mechanism for the ordering of MTs resides in the cortex.

Our study of the developing radial array of MTs in the GCs of *Allium* indicated that, unlike the above systems, the MTs originate from a planar MT-organizing zone located at the central portion of the ventral wall (Marc et al. 1989). In this report we use low temperature, drugs, and centrifugation to show that a) the radial array depolymerizes to a dense layer of MT remnants along the central portion of the ventral wall and subsequent recovery leads to regeneration of the radial array; b) during recovery after a complete depolymerization, which leaves only a diffuse cytoplasmic fluorescence, a dense layer reappears at the ventral wall; and c) the regenerated layer is positioned centrally along the ventral wall, irrespective of the position of the nucleus during the recovery process.

Material and methods

Plant material. Seeds of *Allium cepa* L. cv. White Portugal (Harris Moran Seed Company, Rochester, N.Y., USA) were germinated in moist vermiculite at 24° C as described in Marc et al. (1989). In all experiments we used the cotyledon of 4-d-old seedlings.

Low-temperature treatments. Intact seedlings were enclosed between two layers of a nylon mesh, secured with paper clips, and immersed in a slurry of ice and water. In a cold-room at 4° C and using ice-cold instruments, the terminal portion of the chilled cotyledon was sliced longitudinally in half and a 4–5-mm long segment above the cotyledonary hook was excised. One of the half-cotyledon segments was fixed overnight with an ice-cold fixative consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in a buffer mixture (Marc et al. 1989). The other segment was placed on moist filter paper in an ice-cold Petri dish, transferred to room temperature (24° C), and after a period of rewarming fixed overnight at room temperature.

Treatments with colchicine. Half-cotyledon segments were placed with their cut surfaces on a filter paper saturated with a 100 μM solution of colchicine (Sigma Chemical Co., St. Louis, Mo., USA) in 20 mM 1,4-piperazine diethanesulfonic acid (Pipes) buffer, pH 6.8, in a small Petri dish. The dish was sealed with parafilm and kept for 1 h in the dark at room temperature. Following a rinse with buffer, some segments were fixed as above. The remaining segments were floated on buffer in a small vial and exposed to ultraviolet (UV)-irradiation on the stage of a Zeiss Universal microscope (Carl Zeiss, Oberkochen, FRG) equipped for epifluorescence illumination with a 100-W mercury lamp (HBO 100/W2; Osram, Berlin West, Germany) and a UG-1 excitation filter (365 nm), with the objective lens removed. The segments were then fixed as above.

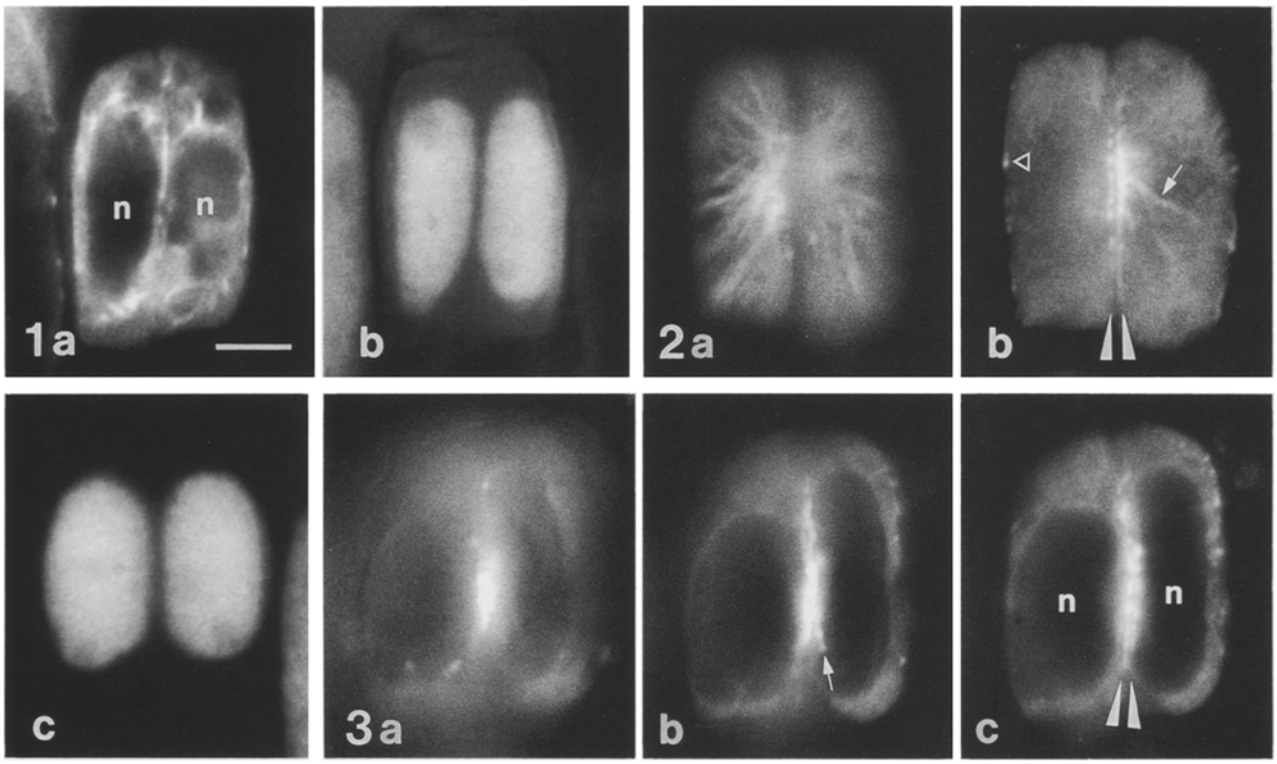
Centrifugation. Half-cotyledon segments were placed with their cut surfaces on a filter paper saturated with a 200 μM solution of colchicine in 20 mM Pipes buffer, pH 6.8, and incubated in a sealed Petri dish for 1 h in the dark at room temperature. The segments were then firmly wrapped in layers of paper towel saturated with the same solution, inserted into a centrifuge tube so that centrifugal force would be directed basipetally, and centrifuged for 15 min at $1000 \cdot g$. The segments were then floated on a buffer solution without colchicine and exposed to UV irradiation as above. In other experiments we used a 200 μM solution of colchicine in buffer as before but also containing 2 μM cytochalasin D (Sigma) and 0.1% dimethyl sulfoxide (DMSO).

Immunofluorescence microscopy. Preparation of epidermal cell layers and processing for tubulin immunofluorescence microscopy were performed as described in Marc et al. (1989). Serum 5051 (a generous gift from Dr. Marc Kirschner, University of California, San Francisco, USA) was diluted 1:60 with phosphate-buffered saline (PBS), and binding was detected by a fluorescein-isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin G (IgG; Sigma) diluted 1:100 with PBS.

Results

Low-temperature treatments. Indications of depolymerization, such as reduced MT frequency and the presence of diffuse cytoplasmic fluorescence, are already apparent after a 30-min exposure to 0° C (details not shown). Although the loss of MTs progressively increases during the first 2–3 h, a complete depolymerization could not be achieved even by a 24-h exposure (not shown). Therefore we limited the treatment to 2.5 h (Figs. 1–3, 4–7).

In GCs that are in an early postcytokinetic stage, as judged by their wedge-shaped nucleus, relatively short and thick MT remnants surround the nuclear surface (Fig. 1). Perinuclear MTs are absent, however, in slightly older (developing) GCs with rounded nuclei (Figs. 2, 3). The extent of depolymerization in these cells varies. In about half of them, MTs are absent from the end walls, their frequency at the dorsal wall and in the inner cytoplasm is greatly reduced, and a diffuse fluorescence is present throughout the cytoplasm (Fig. 2). Nonetheless, some MTs radiating along the inner periclinal wall are still discernible, as well as a layer of MTs along the central strip of the ventral wall, which corresponds to the site of the future pore. In the other half of the population, the array is reduced to MT remnants located mainly along the



Figs. 1–3. Tubulin immunofluorescence in *Allium* GCs exposed to 0° C for 2.5 h. Microtubules adjacent to the central strip of the ventral wall are indicated by pairs of *arrowheads*; *n* nucleus. ×2000; scale bar in Fig. 1 = 5 μm, applies throughout

Fig. 1a, b. Remnants of MTs surrounding the slightly wedge-shaped nuclei in GCs at an early postcytokinetic stage. **a** Median optical section; **b** nuclei stained with Hoechst 33258

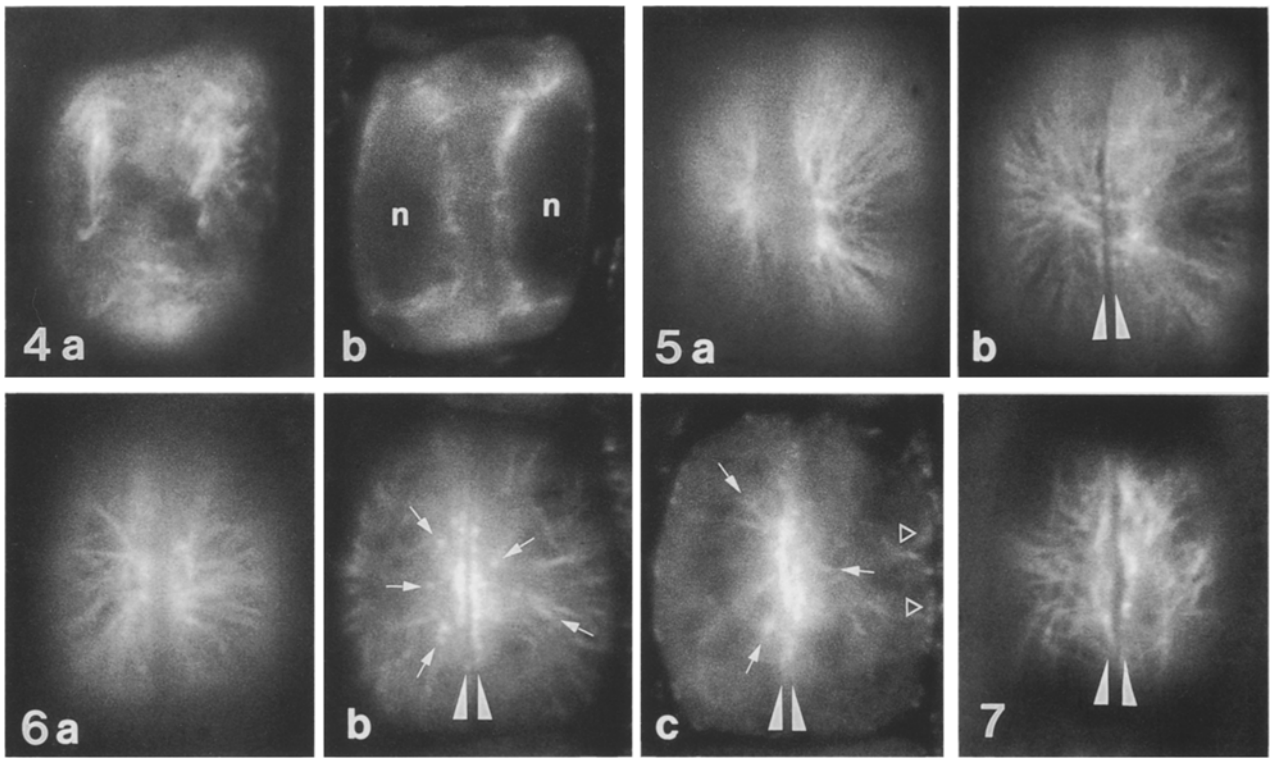
Fig. 2a–c. Remnants of a radial MT array in developing GCs with rounded nuclei. **a** Focus near cell surface. **b** Focus near upper surface of the nuclei; an MT bundle (*arrow*) extends from ventral wall through the inner cytoplasm and only a few MTs remain at the dorsal wall (*triangle*). **c** Nuclei stained with Hoechst 33258

Fig. 3a–c. Serial optical sections through a dense cluster of MTs along the central strip of the ventral wall; *arrow* shows a thick MT stub

central strip of the ventral wall (Fig. 3). Some cells still contain a few thick MT bundles extending from this region into the cytoplasm (Fig. 3b), while in others only a layer of MTs adjacent to either side of the ventral wall remains and persists for 24 h at low temperature (not shown).

Regeneration of MTs occurs very rapidly (within a few minutes) after rewarming the half-cotyledons to room temperature (Figs. 4–7). In GCs in the early postcytokinetic stage, MTs extend from the nuclear surface in random directions throughout the cytoplasm (Fig. 4). Again, perinuclear MTs are absent in developing GCs with rounded nuclei (Figs. 5–7; nuclei not shown). Instead, almost all of these cells contain a radial MT array within 15 min of rewarming, although a diffuse cytoplasmic fluorescence initially remains. Even by 1 min, numerous 'fine' MTs radiating from the pore site along the periclinal wall appear in some cells (Fig. 5). Gradually the density of MTs along the central strip of the ventral wall increases, with many MTs diverging from this site at various angles and extending through the inner cytoplasm (Fig. 6); MTs extend also along the dorsal wall. Relatively thick MT bundles generally reform after longer periods of rewarming (Fig. 7). Although many bundles are disoriented, an overall radial pattern is restored in all cells. Thus, in about half of the GC population (those in which the MTs were initially reduced to small remnants) the recovery involves a complete regeneration of a radial array from the persistent layer of MTs along the ventral wall.

Treatments with colchicine. We employed an anti-MT drug to see whether the depolymerization and reassembly patterns at low temperature could be duplicated by chemical means. Previously we showed that the early postcytokinetic stage lasts only about 20 min, after which MT organization



Figs. 4–7. Tubulin immunofluorescence in *Allium* GCs during rewarming at 24° C following depolymerization for 2.5 h at 0° C. Microtubules adjacent to the ventral wall are indicated by pairs of *arrowheads*. × 2000

Fig. 4a, b. Randomly oriented cytoplasmic and perinuclear MTs in a GC pair at an early postcytokinetic stage, after a 10-min rewarming. **a** Focus near cell surface; **b** median optical section; *n* nucleus

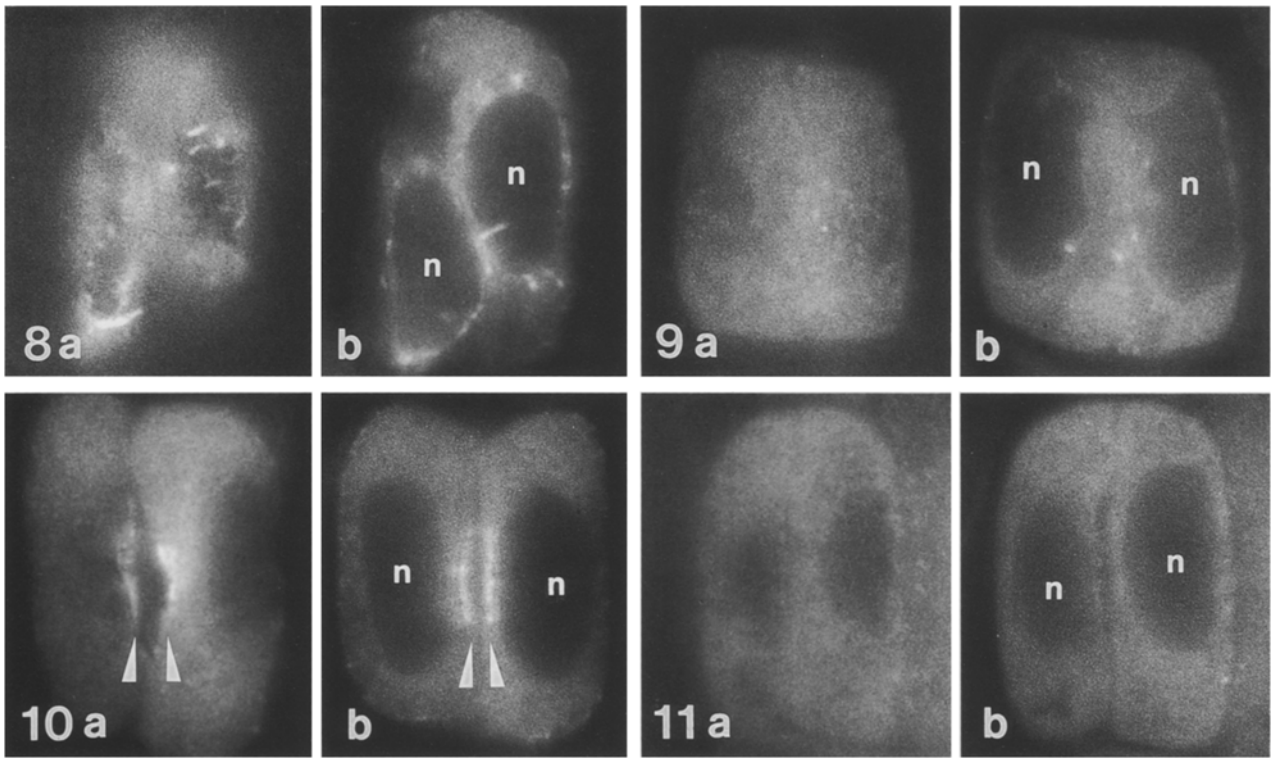
Fig. 5a, b. A radial array of 'fine' MTs in a developing GC pair (past the early postcytokinetic stage), after a 1-min rewarming. **a** Focus at cell surface; **b** focus below cell surface

Fig. 6a–c. Regenerating MTs radiating through the inner cytoplasm (*arrows* in **b**, **c**), after a 1-min rewarming. **a** Focus at cell surface; **b** focus below cell surface; **c** focus near the upper surface of the nuclei, MTs extend along the dorsal wall (*triangles*)

Fig. 7. Disoriented MT bundles, after 10 min of rewarming; focus at cell surface

changes radically as the characteristic radial array becomes established (Marc et al. 1989). In order to avoid confounding results of MT recovery with developmental changes, it is therefore necessary that the effect of the drug be rapidly reversible. Although drugs such as oryzalin or amiprophos-methyl (APM) effectively disrupt plant MTs at very low concentrations (Gunning and Hardham 1982), depending on the dose and the species the effect is either irreversible (Bajer and Molè-Bajer 1986) or requires extended periods of recovery (hours to days; Falconer and Seagull 1987; Venverloo and Libbenga 1987; Cleary and Hardham 1988; Wacker et al. 1988). Colchicine, in contrast, can be deactivated within a few minutes by UV irradiation (Aronson and Inoué 1970; Venverloo and Libbenga 1987).

Colchicine concentrations as low as 10 μM disrupt MT arrays, but the effect is relatively minor. We therefore used a 100 μM solution. A 30-min incubation with this solution produces a strong but variable effect (details not shown), presumably reflecting an incomplete penetration of the drug through the tissue. The effect of 1-h incubation (Figs. 8–19), however, is consistent along each cotyledon and among individual samples. In many cells this treatment causes a complete depolymerization and the accumulation of a uniform, diffuse cytoplasmic fluorescence (Figs. 9, 11), although about one third of the cells still contain remnants of MTs (Figs. 8, 10). In GCs that are in the early postcytokinetic stage, the remnants are associated with the nuclear surface (Fig. 8). In older GCs, in contrast, the remnants are confined to the central strip of the ventral wall (Fig. 10), with the most extensive fluorescence at the mid-region of the strip. The two distinct types of subcellular location



Figs. 8–11. Tubulin immunofluorescence in *Allium* GCs at the early postcytokinetic stage (Figs. 8, 9) and in developing GCs with rounded nuclei (Figs. 10, 11), after a 1-h incubation with 100 μM colchicine; *n* nucleus. ×2000

Fig. 8a, b. Remnants of MTs at nuclear surface. **a** Focus near upper surface of nuclei; **b** median optical section; note wedge-shaped outline of the nuclei (the nuclei may be diagonally positioned because colchicine was introduced during cytokinesis)

Fig. 9a, b. A GC pair showing almost complete MT depolymerization and diffuse cytoplasmic fluorescence. **a** Focus near cell surface; **b** median optical section; note irregular outline of the nucleus

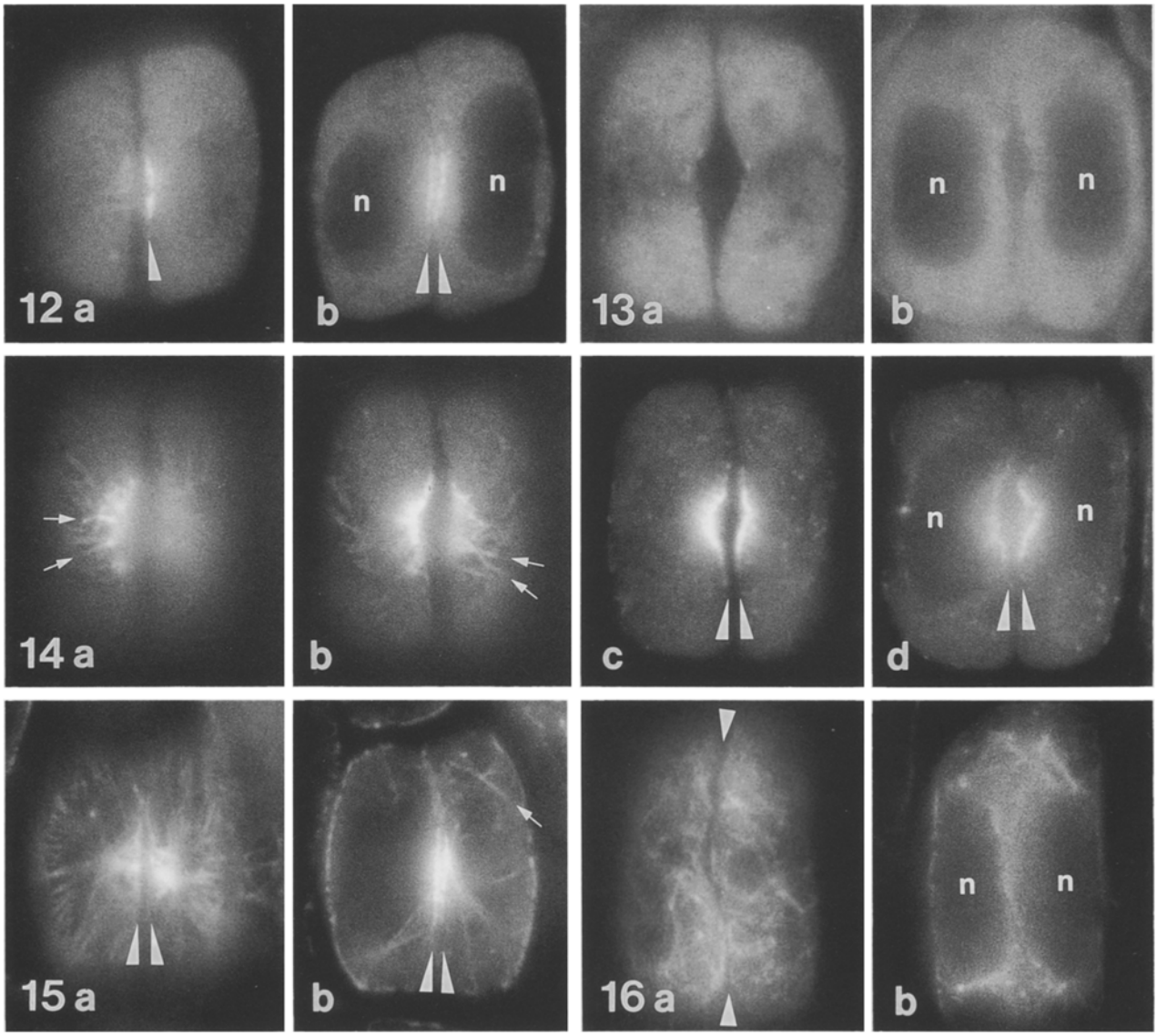
Fig. 10a, b. Remnants of MTs along the central strip of the ventral wall (*arrowheads*). **a** Focus near cell surface; **b** median optical section

Fig. 11a, b. A complete MT depolymerization producing diffuse cytoplasmic fluorescence. **a** Focus near cell surface; **b** median optical section

of the MT remnants are similar to those seen using low temperature; also as before, we have not found cells containing both types concurrently.

Washing the colchicine-treated tissue with buffer for 30–60 min does not by itself lead to notable MT recovery (although it appears to promote recovery induced by UV light). Thus some of the developing GCs still contain remnants of MTs at the ventral wall (Fig. 12), while only a uniform, diffuse cytoplasmic fluorescence remains in most of the cell population (Fig. 13). Irradiation with UV light for 1–3 min, in contrast, induces a substantial recovery, and within 5 min all developing GCs contain various stages of MT regeneration at the ventral wall. In Fig. 14, for example, the regenerating MTs extend from a prominent fluorescent layer along the central strip of the ventral wall toward the dorsal wall. A complete radial array of MTs is eventually regenerated after longer periods of UV irradiation (Fig. 15), although some MTs may appear disoriented. Again, in GCs that are in the early postcytokinetic stage the regenerating MTs extend randomly from nuclear surface throughout the cytoplasm, without any specific association with the ventral wall (Fig. 16). The perinuclear MTs therefore do not contribute to the radial array of cortical MTs.

Colchicine treatments and centrifugation. In order to examine whether the position of the regenerating layer of MTs along the ventral wall is influenced by the nearby nucleus, we displaced the nucleus by a brief centrifugation before the UV-irradiation step. To ensure a complete MT depolymerization in nearly all cells, we doubled the colchicine concentration to 200 μM. Furthermore, because of the additional manipulation associated



Figs. 12–16. Tubulin immunofluorescence in *Allium* GCs with rounded nuclei (Figs. 12–15) and in a GC pair at the early postcytokinetic stage (Fig. 16), during recovery after a 1-h incubation in 100 μM colchicine. Microtubules adjacent to the central strip of the ventral wall are indicated by pairs of *arrowheads*; *n* nucleus. ×2000

Fig. 12a, b. A GC pair after a 30-min wash in buffer, showing remnants of MTs at the ventral wall. **a** Focus near cell surface; **b** median optical section

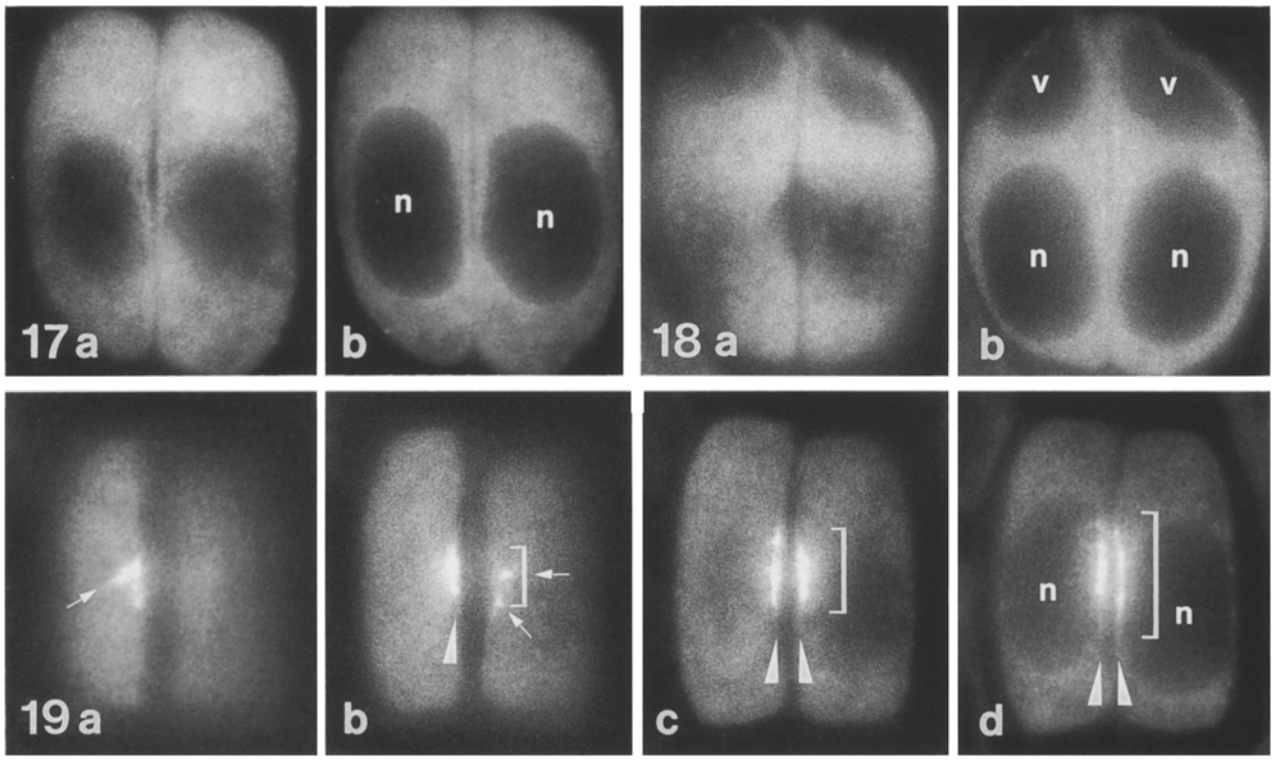
Fig. 13a, b. A GC pair after a 60-min wash in buffer, showing diffuse cytoplasmic fluorescence. **a** Focus near cell surface; **b** median optical section

Fig. 14a–d. Regenerated MTs (*arrows* in **a**, **b**) extending from a prominent layer along the ventral wall, after 5 min of UV irradiation. **a–d** Optical sections at 0, 2, 6, and 10 μm below cell surface (ventral wall in the cell on the right in **d** collapsed somewhat during preparation)

Fig. 15a, b. A complete radial array of MTs, regenerated after a 75-min wash in buffer and 35 min of UV irradiation. Optical sections at 1 and 5 μm below cell surface; *arrow* in **b** indicates a disoriented MT bundle

Fig. 16a, b. Randomly oriented cytoplasmic and perinuclear MTs in postcytokinetic GCs, regenerated after a 30-min wash in buffer and 15 min of UV irradiation. Optical sections at 1 and 9 μm below cell surface; note MTs do not associate specifically with the ventral wall (*arrowheads* in **a**)

with the centrifugation, the total time in the presence of colchicine added up to 1.5–2 h. Consequently, MTs disappeared in almost all cells, leaving only a uniform, diffuse fluorescence throughout the cytoplasm. Thus no MT images can be



Figs. 17–19. Tubulin immunofluorescence in developing *Allium* GCs, following a 1-h incubation with 200 μM colchicine and a brief centrifugation (direction of centrifugal force toward the bottom of the page); *n* nucleus. × 2000

Fig. 17a, b. A complete depolymerization, only diffuse cytoplasmic fluorescence remains. **a** Focus near upper surface of nucleus; **b** median optical section

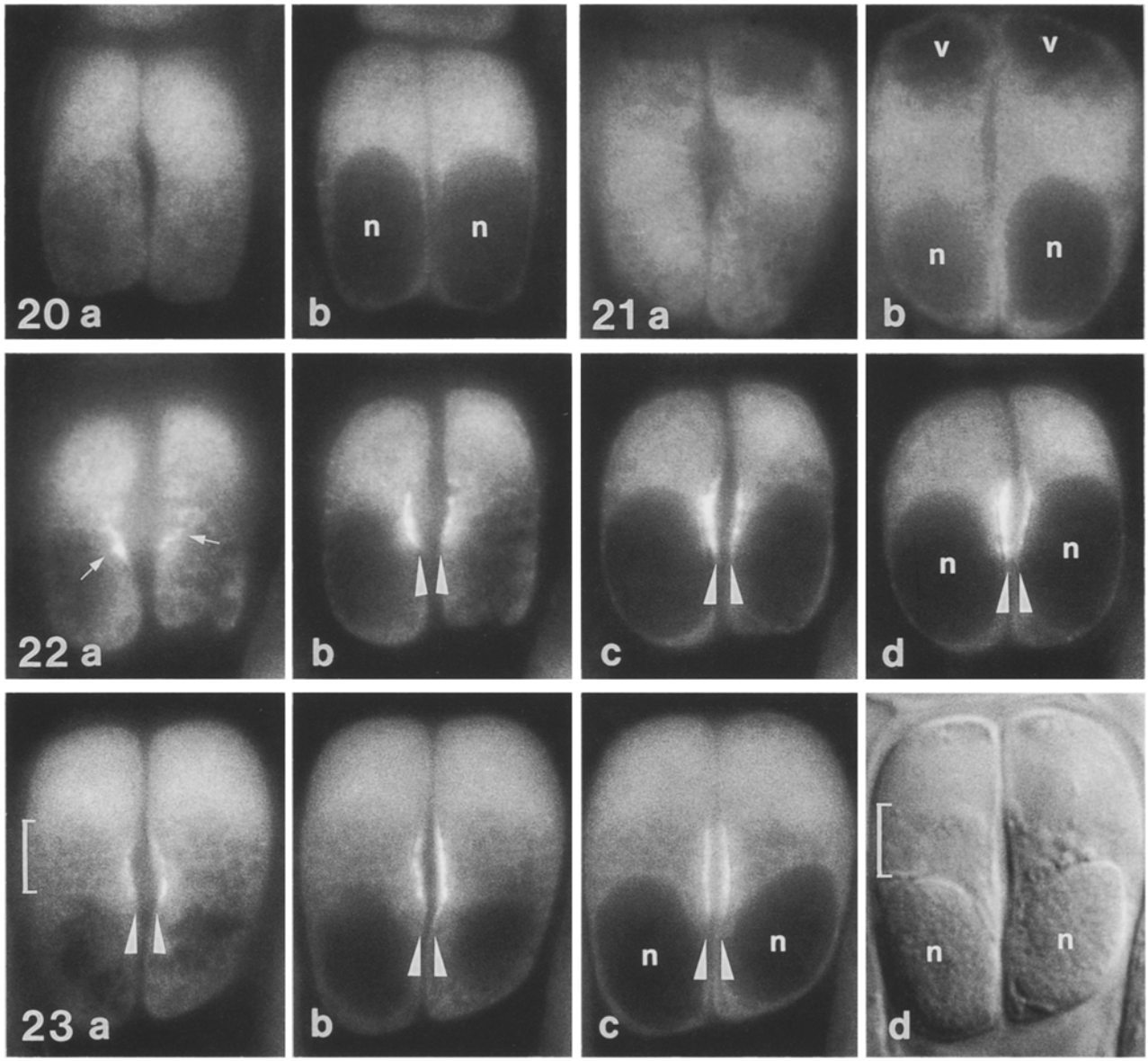
Fig. 18a, b. A complete depolymerization in a GC pair with developing vacuoles (*v*). **a** Focus near upper surface of nucleus; **b** median optical section

Fig. 19a–d. Regenerated MTs (*arrows* in **a**, **b**) extending from a prominent layer along the central strip of the ventral wall (*arrowheads*), after 10 min of UV irradiation. **a–d** Optical sections at 0, 2, 6, and 10 μm below cell surface; note the width of the prominent layer (*brackets*) increases with the depth of focus

found after this treatment in developing GCs (Fig. 17) or even in older GCs with expanding vacuoles (Fig. 18). Nonetheless, within 1–5 min of UV irradiation, MTs again reappear along the central strip of the ventral wall in all developing GCs and gradually extend toward the dorsal wall (Fig. 19). Typically, the prominent layer of regenerated MTs along the central strip is wider at its midregion and narrower toward the periclinal walls. Although in many cells the nucleus is clearly displaced, the regenerated layer of MTs is consistently located centrally along the ventral wall.

Following centrifugation, the position of the nucleus apparently becomes less asymmetric during manipulations preceding fixation (e.g. Fig. 17). We therefore included cytochalasin D, which interferes with organelle movement in the GC (Palevitz and Hepler 1974; Palevitz 1980), in the colchicine solution as well as in the wash buffer during the UV-irradiation step to prevent nuclei from returning to their original position. Depolymerization and regeneration of MTs are similar to those seen before. Thus, while no MT images can be found following the incubation and centrifugation steps either in developing GCs (Fig. 20) or in older GCs with expanding vacuoles (Fig. 21), a prominent layer of MTs reappears at the ventral wall shortly after UV irradiation (Figs. 22, 23). Despite the distinctly asymmetric location of the nucleus, as well as dislocation of smaller cytoplasmic organelles (Fig. 23), the layer of regenerated MTs is again located centrally along the ventral wall.

Binding pattern of serum 5051. From the above results it is apparent that the generation of the characteristic radial array involves a 'switch' from sites of MT initiation at the nuclear surface to the cortex along the ventral wall. Since it has been reported that serum 5051 detects MT-initiation material in root-tip cells of *Allium* (Clayton et al. 1985; Wick 1985a, b), it is of interest to examine



Figs. 20–23. Tubulin immunofluorescence in developing *Allium* GCs after a 1-h incubation with 200 μM colchicine and 2 μM cytochalasin D and followed by a brief centrifugation (direction of centrifugal force toward the bottom of the page); *n* nucleus. ×2000

Fig. 20a, b. A complete depolymerization, only diffuse cytoplasmic fluorescence remains. **a** Focus near cell surface; **b** median optical section

Fig. 21a, b. A complete depolymerization in a GC pair with developing vacuoles (*v*). **a** Focus near cell surface; **b** median optical section

Fig. 22a–d. Regenerated MTs (*arrows* in **a**) and a prominent layer along the central strip of the ventral wall (*arrowhead* pairs), after 5 min of UV irradiation. **a–d** Optical sections at 0, 2, 5, and 9 μm below cell surface

Fig. 23a–d. A prominent layer along the central strip of the ventral wall (*arrowhead* pairs), regenerated after 10 min of UV irradiation. **a–c** Optical sections at 3, 5, and 9 μm below cell surface; **d** differential interference contrast view (same focus as in **c**); *bracket* indicating a layer of sedimented cytoplasmic organelles, appearing as blank spots in **a–c**

whether either or both of the sites of MT initiation are related to the binding pattern of this serum. Irrespective of the developmental stage of the GC, however, the serum binds to small organelles or particles that are distributed randomly throughout the cytoplasm, without any indication of specific association with the nucleus, cell edges, or the ven-

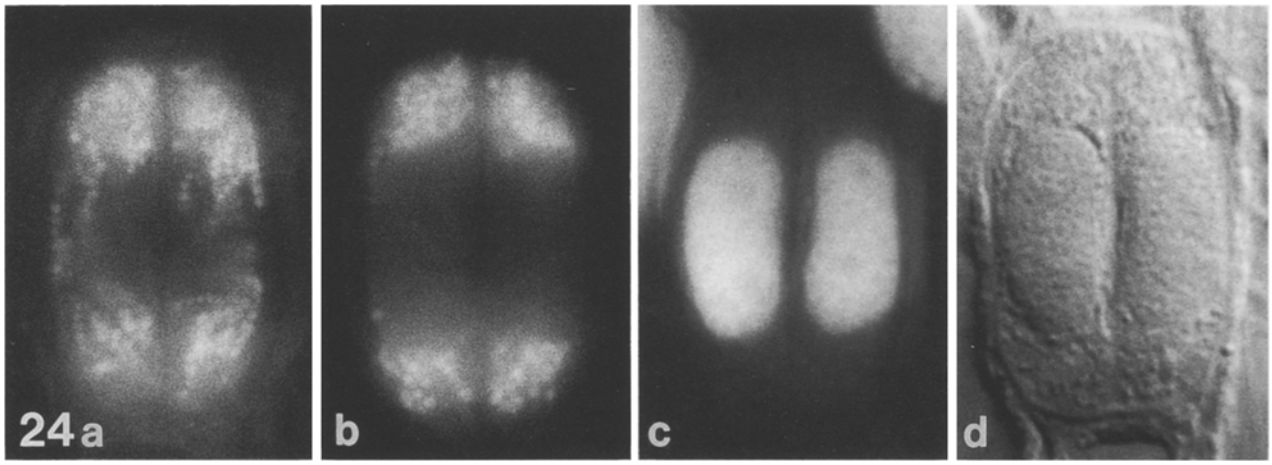


Fig. 24a–d. Binding pattern of serum 5051 (**a, b**) in a developing GC pair of *Allium*. **a** Focus near upper surface of nuclei; **b** median optical section; **c** nuclei stained with Hoechst 33258; **d** differential interference contrast view. ×2000

tral wall (Fig. 24). Similar results were obtained after modifying the fixation and permeabilization procedures, as well as using different dilutions of the serum and different second antibodies.

Discussion

Location of MTOCs. The pattern of partial MT disassembly, whether induced by cold or 100 μM colchicine, falls into two distinct developmental categories. Immediately after cytokinesis, MT remnants are located at the nuclear surface, while starting somewhat later in development the MT remnants form a persistent layer at the ventral wall. It seems likely that the locations of the stable MT remnants coincide with the locations of corresponding MTOCs. Treatments of interphase animal cells with cold or colchicine, for example, show that MTs in the centrosomal region, which acts as an MTOC, are more resistant to depolymerization than those in the peripheral cytoplasm (Osborn and Weber 1976).

During recovery after partial MT disassembly in GCs, the regenerated MTs show two distinct types of organization, which correspond to the two developmental stages mentioned above. Thus the MTs either extend from the perinuclear region or radiate from the cortical cytoplasm adjacent to the central portion of the ventral wall. Microtubule reassembly therefore occurs at specific subcellular locations. Moreover, because the two types of organization follow each other rather than being present at the same time in the cell, the subcellular location of MT initiation apparently 'switches' (see below). It is therefore unlikely that MTs that originate in the perinuclear region are major contributors to the radial array, reinforcing a similar conclusion made on the basis of detailed study of changes in MT organization during normal GC development (Marc et al. 1989). This conclusion is further strengthened by the continuous generation of new MTs later in development, long after nuclear-associated tubulin immunofluorescence has disappeared (Marc et al. 1989).

Depolymerization induced by 200 μM colchicine results in the total disappearance of recognizable MTs in almost all cells, leaving only diffuse immunofluorescence distributed throughout the cytoplasm. Nonetheless, during recovery a dense layer of MTs consistently reappears in developing GCs along the central strip of the ventral wall, even if the nucleus has been displaced by centrifugation, with or without the presence of cytochalasin D. The nucleus therefore has no effect on the placement of the regenerated layer, presumably because by this stage of development the position of the relevant MTOCs has been fixed and cannot be altered by displacing the nucleus. That cytochalasin D itself seems to have no effect is interesting, because it indicates that F-actin-dependent processes are not involved in the generation of the radial array. This result also complements previous findings (Palevitz and Hepler 1976).

It is apparent from the pattern of partial disassembly by cold or 100 μM colchicine that, within the central strip of the ventral wall, the layer of persistent MT remnants concentrates near the midregion rather than at the edges where the ventral wall joins the periclinal walls. Likewise, during recovery after complete depolymerization by 200 μM colchicine, a dense layer is again regenerated at a similar location. Although these experiments do not eliminate the possibility that minute lengths of MTs, imperceptible by immunofluorescence mi-

croscopy, remain and subsequently act as seeds for MT reassembly, if present such seeds clearly do not accumulate at cell edges. Instead, the regenerated layer is typically widest at its mid-region. Furthermore, the reestablishment of this layer precedes the outgrowth of MTs toward the adjacent walls. Microtubule generation therefore first appears, and henceforth concentrates, at the mid-region of the central strip of the ventral wall, thus forming a planar MT-organizing zone. As also shown previously (Marc et al. 1989), growth of MTs originating from this zone then progresses outward along the adjacent walls, thus generating the characteristic radial array (compare Galatis 1980; Galatis et al. 1983; Busby and Gunning 1984).

Origin and properties of the MT-organizing zone. The MT-organizing zone presumably represents an aggregation of MTOCs in the cortex adjacent to the corresponding region of the ventral wall. The identity of the MTOCs, however, remains unknown. Despite the superior preservation of the three-dimensional aspect of the GCs relative to cells obtained by squashing *Allium* root tips followed by air-drying (Clayton et al. 1985; Wick 1985a, b), the material detected by serum 5051 fails to co-localize with the stable MT remnants or with the sites of MT regeneration, either in the perinuclear region or in the cortex. Hence the 5051 immunoreactive material appears to be irrelevant regarding MTOCs in the GC.

Although active MTOCs must be present in the MT-organizing zone from the beginning of its inception, without the use of a specific probe their origin and identity are unclear. Since the ventral wall is a newly formed structure, and MT initiation concentrates at its mid-region rather than at the edges, a process of activation of preexisting MTOCs (Gunning et al. 1978; Galatis et al. 1983; Apostolakos and Galatis 1985) is unlikely unless it involves re-deployment of MTOCs from another location such as the antecedent phragmoplast. One possibility is that the MTOCs are newly created at the zone. Alternatively, active MTOCs could be relocated to the zone from the proximal side of the telophase nucleus, presumably after reformation of the nuclear envelope has been completed. Results from the centrifugation experiments indicate that in rare cases, cortical MTs at the ventral wall in young GCs first appear opposite the displaced nucleus rather than at the central strip (details not shown). Whether this indeed represents a relocation of MTOCs from the displaced nucleus or simply a distortion of a symmetrical but unconsolidated wall-MTOC complex by the centrifugal force is difficult to ascertain, however, mainly because the frequency of GCs at the relevant developmental stage is generally low in MT-depolymerization experiments. Whatever mechanism is involved, it is apparently accomplished within about 20 min after the completion of cytokinesis, as this is the duration of the 'postcytokinetic' stage after which the generation of the radial MT array begins (Marc et al. 1989).

Since regeneration of MTs at the MT-organizing zone occurs also in older, vacuolated cells, the zone apparently retains its MT-initiating potential well into the cell-expansion stage. Hence the generation of the entire radial array during normal development is likely to be the product of continuous initiation in the zone, rather than the result of a sequential activation of MTOCs at discrete cortical locations, a mechanism proposed for the organization of MTs in the guard mother cell of *Zea* (Galatis 1982) and the air chamber of *Marchantia* (Apostolakos and Galatis 1985). Nonetheless, the total number of MTs in the radial array increases as it keeps pace with cell expansion, and some MTs generated in the later stages of development are oriented periclinally along the ventral wall, rather than anticlinally as in the majority of MTs in the radial array (Marc et al. 1989). Thus some modifications in the MT-initiation process apparently occur within the zone during the development of the GC. It has been shown that the number of MTs nucleated by animal centrosomes increases in proportion to the concentration of free tubulin (Mitchison and Kirschner 1984). Comparable information in higher plants is unavailable, but it is known that levels of total tubulin increase during development in carrot embryos (Cyr et al. 1987). Details of the operation of such factors in GC differentiation remain to be established.

The question arises whether the MTOCs that constitute the MT-organizing zone regulate aspects of organization other than facilitating MT initiation and defining the location of their origin. As described above, the pattern of MT regeneration after depolymerization indicates that MTs originating at the zone grow distally. Thus the zone also defines the direction of MT growth. In this regard the zone appears to be analogous to the fibroblast centrosome and the interphase array it generates (Osborn and Weber 1976; Mitchison and Kirschner 1984; Soltys and Borisy 1985), as well as erythrocyte centrioles which give rise to a characteristic marginal MT band (Nemhauser et al. 1983; Murphy et al. 1986). In both examples the direction of growth is also matched by structural polarity of the MTs (Euteneuer et al. 1985; Kirschner and Mitchison 1986; see also Mogensen et al. 1989).

We thank Dr. Marc Kirschner for the 5051 serum and Ms. Elizabeth Bruce for printing the figures. This research was supported by funds from the University of Georgia Center for Plant Cell and Molecular Biology, and National Science Foundation grants DCB-8703292 to B.A.P. and DCB-8803286 to B.A.P. and J.M.

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Received 11 May; accepted 18 July 1989