Control of division plane in normal and griseofulvin-treated microsporocytes of *Magnolia*

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Summary

Meiotic cytokinesis in microsporocytes of *Magnolia* is an unusual form of the simultaneous type; phragmoplast expansion is not accompanied by a cell plate, wall deposition is centripetal, and infurrowing of the cytoplasm after first meiosis results in semicells connected by an isthmus. Dyad domains are further defined by interaction of extensive radial systems of microtubules emanating from the daughter nuclei and by a band of organelles polarized in the equatorial region. After second meiosis, phragmoplasts are organized in the interzonal regions between the sister nuclei in each semicell and also at the interfaces of microtubules forming secondary interzona between non-sister nuclei. Wall deposition is not initiated until after phragmoplasts expand to the cell periphery and fuse in the isthmus. Centripetal wall deposition in boundaries of spore domains marked by radial arrays of microtubules results in simultaneous quadripartitioning of the microsporocyte into a tetrad of microspores. Treatment of microsporocytes with griseofulvin resulted in atypically placed nuclei and supernumerary nuclei. Abnormalities could be traced to displaced spindles and to spindles with multiple poles. Drug-induced multinucleate coenocytes were able to organize microtubules and initiate cytokinesis in altered patterns. The data suggest that spindle alignment and aggregation of spindle poles are two components of spatial control that are operative in determining the normal arrangement of nuclei, and that the final placement of walls is a function of the postmeiotic nuclear-based radial arrays of microtubules which define spore domains.

Key words: cytokinesis, *Magnolia*, microtubules, microsporogenesis, phragmoplast, pollen.

Introduction

The cytokinetic apparatus involved in quadripartitioning the microsporocyte into a tetrad of spores differs significantly from that of vegetative cell division (Brown and Lemmon, 1991a, 1992). In histogenesis, the cytokinetic apparatus consists of the preprophase band of microtubules (PPB) and the phragmoplast (Gunning, 1982; Wick, 1990, 1991). The PPB is a girdling band of microtubules that marks the site where the new wall will join parental walls after mitosis. The new wall is deposited in association with centrifugal expansion of the phragmoplast, a complex cytoskeletal organelle consisting of microtubules, co-aligned F-actin, endoplasmic reticulum and associated vesicles.

In meiosis, no PPB marks the future division sites, the role of the phragmoplast is unclear, and the course of wall deposition may be centripetal rather than centrifugal. Meiotic cytokinesis may occur successively after each nuclear division, or the four spores may be cleaved from the common cytoplasm simultaneously after meiosis. While cell plates typically expand centrifugally in association with phragmoplasts in successive cytokinesis, there is considerable variation in the course of wall deposition in simultaneous cytokinesis (Waterkeyn, 1962; Sampson, 1969; Brown and Lemmon, 1991b,c). In most organisms there is at least some degree of cytoplasmic infurrowing. This infurrowing is a plant-like process (as opposed to infurrowing in animal cells) resulting from localized deposition of wall material initiated at the periphery of the microsporocyte.

The role of the phragmoplast in cytokinesis by infurrowing is unclear. Several recent studies have reported the absence of phragmoplasts after first nuclear division in tomato (Hogan, 1987), honeysuckle (Brown and Lemmon, 1988), eggplant (Traas et al., 1989), and *Gasteria* (van Lammeren et al., 1985). In all of these examples, a complex of phragmoplasts develops after second division and simultaneous cytokinesis results from cell plates, infurrowing, or some combination of the two processes. From studies of orchids with simultaneous cytokinesis, we know that typical phragmoplasts can be formed after first meiosis without the deposition of wall material, or with only the formation of a floating disc of wall material that is later resorbed (Brown and Lemmon, 1991b). Thus, it appears that the phragmoplast proper can be independent of the formation of a cell plate.

An early study of microsporogenesis in *Magnolia* using paraffin sectioning techniques (Farr, 1918) reported an "equatorial spindle" (phragmoplast) after first meiosis, the
absence of a cell plate, and subsequent partial infurrowing. Following second meiosis, secondary arrays of “fibers” are re-established across the equatorial region of first meiosis. Again, no cell plates form, and the microspores are cleaved by infurrowing. This suggests an uncoupling of events that are usually tightly coordinated, i.e. cytokinesis with karyokinesis, and cell plate deposition with phragmoplast expansion. The isolation of these events might provide some clues to the roles of components of the cytokinetic apparatus in the control of division plane and wall development.

On the basis of comparative studies of sporogenesis, we put forward the hypothesis that the direct control of division plane in microsporogenesis is a function of nuclear-based radial arrays of microtubules that define spore domains. The cytoplasmic domain model (Brown and Lemmon, 1992) accounts for cleavage of spores in a pattern that reflects the position and number of nuclei. In this model it is assumed that spindle alignment, and therefore final arrangement of nuclei, is closely regulated, since many plants produce spore tetrads in highly predictable patterns.

The relationship of cytoplasmic infurrowing to nuclear-based microtubules and phragmoplasts in meiotic cytokinesis is the subject of the present investigation. In microsporocytes of *Magnolia*, as in other plants where wall deposition is wholly centripetal, the spatial control of division plane is unclear. We were particularly interested in learning whether or not typical phragmoplasts are formed after first and second meiosis, and whether or not any guiding cytoskeletal elements mark the path of the subsequent wall ingrowths. As a means of testing the role of cytoplasmic domains in control of division plane, we treated microsporocytes with drugs known to perturb normal organization of microtubules. While the drugs griseofulvin and CIPC are known to cause multipolar spindles and faulty distribution of chromosomes in plant mitosis (Vogelmann et al., 1981; Clayton and Lloyd, 1984), little is known of their effect on meiotic cells. CIPC was shown to disturb meiosis in microsporocytes of eggplant, but perinuclear radial microtubule systems were severely disrupted and phragmoplasts did not form (Traas et al., 1989). In the present study, griseofulvin proved to be most useful in that it allowed meiosis and cytokinesis to proceed, but altered the alignment of spindles and/or the number of nuclei resulting from meiosis.

**Materials and methods**

Flower buds of the cultivated Japanese magnolias *Magnolia tripetala* and *Magnolia denudata* used in this investigation were collected on the USL Campus. No differences were detected in the process of microsporogenesis in the two species. The buds treated experimentally with drugs were all of *M. denudata*. Griseofulvin was put into solution in dimethyl sulfoxide (DMSO) at 100× final concentration. The final concentration of DMSO was less than 1% of the drug solution.

The flowers of *Magnolia* contain numerous anthers in a graded developmental series; the outermost being the most mature. The stage of development was estimated from measurements of the flower buds and verified by an aceto-orcein squash of a portion of an anther. For experimental treatment, flowers were selected with the outer whorl of anthers in prophase. The flower bud was then placed upright in a vial with peduncle immersed in drug solution. Another flower bud was placed in a vial of distilled water to serve as a control. The vials were covered by inverting a beaker over them to retard evaporation. Development was monitored by periodically removing an anther through a small window cut in the perianth with a fine razor blade. After 22 hours, 90% of the microsporocytes in the control had progressed to dyad or tetrad stage of development. Development in buds treated with 10 µM griseofulvin was comparable to that of controls, but many abnormalities resulted. Anthers from controls and drug-treated buds were removed and thinly sliced with a razor blade into a drop of fixative.

For indirect immunofluorescence microscopy of tubulin/microtubules, cells were fixed in 4% formaldehyde in a microtubule-stabilizing buffer, digested in an enzyme mixture, extracted in Triton X-100, and labeled with FITC-conjugated secondary antibody to anti-tubulin according to methods previously published (Brown and Lemmon, 1988). For correlation with nuclear stages, some preparations were counterstained with propidium iodide. All preparations were mounted in Mowiol with 0.1% phenylenediamine to retard fading. Preparations were studied and photographed with a BioRad Confocal Laser Scanning Microscope (CLSM).

**Results**

The meiotic spindle of *Magnolia* is broad with distinct kinetochoore bundles and pole-to-pole non-kinetochore micro-

**Figs 1-8.** Normal meiosis I in microsporocytes of *Magnolia*. Microtubules stained by indirect immunofluorescence. Bar, 10 µm.

**Fig. 1.** Metaphase I. The spindle, which is located in the center of the cell, consists of conspicuous kinetochore and non-kinetochore fibers terminating in broad polar regions.

**Fig. 2.** Anaphase I. Kinetochore fibers have shortened. Thickening of non-kinetochore fibers is first observed at periphery of the spindle. Additional microtubules radiate from the polar regions. (A) Surface; (B) optical mid-section.

**Fig. 3.** Telophase I. The phragmoplast originates in the interzonal spindle between groups of chromosomes. Microtubules extend from proximal surfaces of the reforming nuclei. Dark regions marking the interface of opposing sets of microtubules are initially staggered. Microtubules proliferate at the polar regions.

**Fig. 4.** The early phragmoplast. Microtubules become organized into distinct bundles that taper away from non-staining mid-plane. Microtubules radiate from the nuclear envelopes.

**Fig. 5.** The phragmoplast expands centrifugally. (A) Surface view showing the late phragmoplast and systems of microtubules radiating from nuclei. (B) Optical mid-section showing that radial microtubules remain in center of the sporocyte after the phragmoplast has expanded into a hollow ring at the periphery of the cell. (C) Chromosomes and organelles stained by propidium iodide. Condensation of chromosomes indicates that the sister nuclei have entered prophase II. Although no dyad wall is deposited, the concentration of organelles in the equatorial region effectively separates the cell into two domains.

**Fig. 6.** Fully expanded phragmoplast. The dark mid-plane bisecting bundles marks the region where infurrowing will occur.

**Fig. 7.** Radial systems of microtubules emanating from nuclei replace the phragmoplast.

**Fig. 8.** By prophase II, cytoplasmic infurrowing has resulted in semi-cells connected by an isthmus. (A) Optical section showing microtubules felting the elongating nuclei. (B) Optical mid-section showing that the arrays of radial microtubules persist in the isthmus, but are only indirectly connected to the nuclei through a reticulum of microtubules.
tubules (Fig. 1). As the chromosomes move apart in early anaphase, the bundles of non-kinetochore microtubules become thickened, and astral-like microtubules proliferate at the broad polar regions (Fig. 2). Proliferation of microtubules in the equatorial region initiates the interzonal apparatus consisting of bundles of microtubules between the two groups of chromosomes (Fig. 3). At the periphery of the interzonal apparatus the microtubules radiating from the two groups of chromosomes are less bundled. As the nuclear envelopes re-form there is a decrease in the astral microtubules and the early phragmoplast is organized in the interzonal region (Fig. 4). The phragmoplast, which consists of fusiform bundles bisected by a dark zone, becomes shorter than the initial interzonal apparatus (seen in Fig. 3) and takes on a barrel-like shape (Fig. 4). As the phragmoplast expands beyond the interzone the brush-like bundles of microtubules no longer extend to the nuclei (Fig. 5). The phragmoplast expands along the interface of opposing radial systems of microtubules emanating from the daughter nuclei and reaches the periphery of the cell (Figs 5, 6), but no wall is deposited (Figs 5-7). A conspicuous system of microtubules radiating from the proximal surfaces of the nuclei remains after the phragmoplast disappears (Fig. 7). Interaction of the two radial systems of microtubules in the equatorial region, as well as the polarization of organelles into an equatorial band (Fig. 5) effectively marks the dyad domains despite the lack of a dyad wall. Instead of centrifugal wall deposition in association with the phragmo-
plast, the new wall is initiated after the phragmoplast reaches the periphery.

Deposition of the new wall begins at the cell surface (Fig. 7) and proceeds centripetally along the interface of opposing systems of microtubules radiating from the daughter nuclei. Continued ingrowth of the wall during inframeiotic interphase frequently results in semicells connected by a central isthmus at prophase II (Fig. 8). Remnants of the radial microtubules remain in the isthmus, but an irregular reticulum of microtubules becomes intercalated between them and the nuclear envelopes (Fig. 8).

The crescent-shaped prophase II nuclei are felted with microtubules that appear to focus at multiple points in the perinuclear areas (Fig. 8). By metaphase II, all endoplasmic microtubules have disappeared (Fig. 9). Phragmoplasts between the sister groups of chromosomes are initiated and completed (Figs 10-12) in the same manner as described for first meiosis. Microtubules radiating from the four nuclei interact to establish secondary interzonal arrays in the equatorial region of first division (Fig. 11). Individual phragmoplasts initiated in the mid-planes of each of these interzonal arrays (Fig. 11) expand and fuse to re-establish a phragmoplast complex in the first division equator (Fig. 12). The arrangement of the phragmoplast complex is dependent upon the arrangement of nuclei in the sporocyte: pairs of nuclei in the semicells may be side-by-side (tetragonal arrangement, Fig. 11) or at right angles (decussate arrangement, Fig. 12). In Fig. 12, one primary phragmoplast is seen in equatorial view while the other is in polar view; the secondary phragmoplast in the isthmus is somewhat oblique. Again, cytokinesis is centripetal; wall deposition at the periphery of the dyad domains results in ingrowth (Fig. 13). Continued ingrowth in all cytokinetic planes eventually quadripartitions the microsporocyte (Fig. 14). Cleavage of the isthmus is somewhat irregular and can result in unusual configurations (e.g. see Fig. 14). The

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**Figs 9-15. Normal meiosis II and simultaneous cytokinesis. Bar, 10 µm.**

**Fig. 9.** Metaphase II. The spindles form simultaneously in each semicell.

**Fig. 10.** Telophase II. Development of interzonal arrays between sister nuclei.

**Fig. 11.** Secondary interzonal arrays between non-sister nuclei are initiated by interaction of microtubules radiating from nuclei lying in a single plane (tetragonal arrangement).

**Fig. 12.** Phragmoplasts interconnecting four nuclei in decussate arrangement.

**Fig. 13.** Phragmoplasts expand to the periphery of the cell. (A) Surface view showing initiation of wall ingrowths between sister nuclei. (B) Optical mid-section showing reorganization of a phragmoplast in the first division site (compare with Figs 9 and 10).

**Fig. 14.** Wall ingrowths in all cytokinetic planes accomplishes quadripartitioning of the microsporocyte into a tetrad of microspores. Microtubules are organized into nuclear-based radial arrays.

**Fig. 15.** After cytokinesis, each microspore becomes ovoid with radial microtubules extending equally from around the perimeter of the elongate nuclei.
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Radial systems of microtubules persist in the young microspores (Fig. 15).

Callose is not deposited in association with the expanding phragmoplast but is seen as a peripheral ring in the equatorial region after first meiotic division (Figs 16, 17). The intersporal septa that isolate the tetrad of spores following meiosis are callosic (Figs 16, 18).

Drug treatments

Prolonged treatment with 10 µM griseofulvin resulted in coenocytes with abnormally placed nuclei (Fig. 19) and multiple nuclei (Fig. 20). Fig. 19 shows a sporocyte with two nuclei in the isthmus region (the first division equatorial region) where cytokinesis would normally occur. Multiple nuclei appear more or less evenly spaced as a result of the development of radial perinuclear microtubule systems (compare with microtubules shown in Fig. 26). Confocal microscopy of stained microtubules revealed abnormalities in alignment and organization of spindles. In metaphase I, the spindle was often acentric in the cell as well as multipolar (Fig. 21). A frequently observed configuration was a conical spindle with one pole ridged and the other splayed into a circle with numerous poles (Fig. 22). In microsporocytes with conical spindles the chromosomes appeared to be arranged in a contorted ring.

Similar abnormalities in alignment and organization of second division spindles (Fig. 23) resulted in arrangements of nuclei not seen in normal meiosis and in tetrads with supernumerary nuclei. Whereas tetrads are normally either decussate or tetragonal, both linear tetrads (Fig. 24) and T-shaped tetrads (Fig. 25) resulted from griseofulvin treatment. Treated microsporocytes were able to organize radial microtubule systems and phragmoplasts in the presence of griseofulvin (Figs 24-27). Even when microsporocytes contained as many as 6-10 nuclei, radial systems of microtubules were organized around each nucleus (Fig. 26). Phragmoplast-like arrays were triggered in the interfaces of opposing microtubule systems and infurrowing initiated division of the cytoplasm in the newly determined cytokinetic planes (Fig. 27).

Discussion

Unlike histogenesis, where a PPB marks the division site before mitosis, the spatial control of cytokinesis in sporo-
genesis involves a radial perinuclear microtubule system. The distal ends of these radial microtubules define boundaries of cytoplasmic domains around each nucleus in the microsporocyte. Phragmoplasts are triggered in these boundaries and new walls are deposited between adjacent domains. In microsporocytes of *Magnolia*, typical phragmoplasts form after each nuclear division, but walls develop centripetally. Thus, the process of phragmoplast development and cell plate deposition are uncoupled. Importantly, some degree of infurrowing following first meiosis results in semicells joined by an isthmus that provides an absolute marker of the first division plane. These features are used in analysing components of the cytokinetic apparatus in the quadripartitioning of sporocytes lacking PPBs.

Figs 21-27. Microtubule arrays in abnormal meiosis following treatment of microsporocytes with griseofulvin. Bar, 10 µm.
Fig. 21. Metaphase I. The abnormal spindle is multipolar and is positioned acentrically in the cell.
Fig. 22. Metaphase I. A conical spindle with one half-spindle forming a ridge-like polar region and the other half-spindle fanning into numerous poles.
Fig. 23. Metaphase II. Two views of the abnormal spindles, each with several polar regions. In this cell, the distribution of chromosomes was apparently normal in the preceding meiosis I. Perturbation frequently resulted in more than two groups of chromosomes at the start of meiosis II.
Fig. 24. Linear arrangement. Normal phragmoplasts are located between sister nuclei, but a phragmoplast is absent between the non-sister nuclei in the interior of the microsporocyte.
Fig. 25. T-shaped arrangement of nuclei with normal phragmoplasts in altered arrangement.
Figs 26-27. The method of cytokinesis in multinucleate coenocytes induced by drug treatment appears to be identical to that in untreated cells.
Fig. 26. Radial systems of microtubules emanating from each nucleus result in equidistant positioning of nuclei. Phragmoplasts form in all zones of interaction among opposing arrays of microtubules.
Fig. 27. Wall ingrowth (arrows) is initiated in the mid-planes of phragmoplasts at the periphery of the cell and proceeds inward to divide multinucleate coenocytes.
In simultaneous cytokinesis, phragmoplasts are organized in the interzonal regions between sister nuclei and in the secondary interzonal spindles that form between non-sister nuclei (Waterkeyn, 1962; Heslop-Harrison, 1971; Brown and Lemmon, 1992). In both cases, pre-existing systems of microtubules serve as a framework for the higher level of organization typical of phragmoplasts (Schopfer and Hepler, 1991). The phragmoplast originates from the interzonal array and develops into a compact, highly ordered array consisting of short brush-like microtubules on either side of an unstained dark zone. The dark zone occurs where the distal (+) ends of microtubules overlap (Gunning, 1982; Euteneuer et al., 1982; Vantard et al., 1990; Asada et al. 1991). A recent critical study of F-actin in the phragmoplast revealed a corresponding sharp demarcation of rhodamine-phalloidin staining on either side of the dark line (Schopfer and Hepler, 1991).

Although the meiotic phragmoplasts of Magnolia appear similar to mitotic phragmoplasts in structure and dynamics, they are very different with respect to their involvement in cell plate deposition. Phragmoplast expansion to the periphery of the cell is not accompanied by cell plate deposition, and new walls begin as girdling ingrowths. Dyad walls are initiated after telophase I but are not completed; the typical sporocyte in second meiosis appears as two semicells connected by a central isthmus. Very similar development, in which a prominent ring partially divides cytoplasm into a dyad after first division, has been reported in the cycad Stangeria (Rodkiewicz et al., 1988) and the primitive angiosperm Laurelia (Monimiaceae) (Sampson, 1969). In a few isolated examples, continued infurrowing may result in complete cytokinesis into a dyad before meiosis II as in some Magnolia (Gabara, 1971) and Asimina (Annonaceae) (Locke, 1936).

The dyad domains are further marked by a band of organelles. The organelle band is a widespread feature in sporogenesis (for review see Brown and Lemmon, 1991a). Tanaka (1991) has presented evidence that radial perinuclear microtubule systems function in the orientation of the plastids during microsporogenesis in Lilium. The formation of cytoplasmic domains typically involves apportionment of cytoplasmic organelles in association with the radial perinuclear microtubule system (see Menzel and Elsner-Menzel, 1990; and Brown and Lemmon, 1992, for discussion). It has further been suggested that the radial perinuclear microtubule system sweeps a system of F-actin to the equatorial region (Traas et al., 1989; Tanaka, 1991) and that a disc of F-actin marks the first division site in egg plant (Traas et al., 1989). It would appear that the boundary between dyad domains, while real enough, does not serve as an absolute marker of a division site for quadrification of the tetrad. As will be discussed later, when spindle alignment is disrupted by drug treatment, second division nuclei are often placed in the isthmus.

Phragmoplasts formed after second meiosis expand to the periphery of each semicell of the “dyad” and are followed by centripetal wall deposition. Microtubules reappear in the isthmus after second division but not simply in the same pattern as the microtubules after first division. Radial systems of microtubules emanating from the four nuclei interact in the isthmus and give rise to individual phragmoplasts which fuse into a single phragmoplast. Wall deposition in the isthmus is variable, with walls initiated at the peripheries of the intersecting phragmoplasts. Thus, the completion of cytokinesis in the first division equator appears to be typical of simultaneous cytokinesis in other plants, in spite of the extreme degree of infurrowing in the first division equatorial plane.

The role of the phragmoplast in centripetal wall deposition remains enigmatic. It is possible that it is vestigal, transports wall precursors, or lays down some structure that marks the division plane. The function of phragmoplasts in microsporocytes of Magnolia could be similar to that in histogenesis where the phragmoplast is thought to transport vesicles to the site of wall deposition (Gunning, 1982; Schopfer and Hepler, 1991). TEM studies of Magnolia have shown that vesicles are concentrated in the undivided equatorial region (Gabara, 1971). It may be only the timing and course of wall development that is altered in centripetal infurrowing.

Results of drug studies provide important clues to the components of spatial control of cytokinesis in microsporogenesis. The regularity with which most plants produce tetrads in species-specific patterns has long been taken as evidence for the existence of factors that determine spindle alignment (Heslop-Harrison, 1971). In the present study griseofulvin treatment upset the orderly process of division, but allowed development of phragmoplasts which were abnormally positioned but otherwise normal in appearance and function.

Normal tetrads of Magnolia are either decussate or tetragonal (Farr, 1918; present study). Both the arrangement and number of microspores cleaved from the treated microsporocytes were abnormal. These abnormalities could be traced to displaced spindles and to spindles with multiple poles. It is thought that griseofulvin affects microtubule associated proteins (MAPs) rather than directly acting on polymerization of microtubules (Roobol et al., 1977). It may be that target MAPs play a role in spindle alignment and spindle pole consolidation through positioning and consolidation of microtubule organizing centers (MTOCs). The development of spindle polarity is thought to involve recruitment and consolidation of cytoplasmic proteins (Leslie, 1990). This process is of particular importance in plant cells where the spindle poles are diffuse structures.

Griseofulvin frequently induced T-shaped or linear coenocytes that were capable of initiating and completing cytokinesis. The resultant tetrads, although unusual in arrangement, appeared normal in other respects. Many microsporocytes were seen in which nuclei were located in the isthmus. This suggests that following infurrowing after first division there is no residual structure or memory of the first division site. Drug-induced multipolar spindles resulted in abnormal “tetrads” with supernumerary spindles. Abnormal tetrads with extra nuclei were able to organize perinuclear radial microtubules. Interaction of the opposing microtubule arrays defined sites of phragmoplast development and subsequent cytoplasmic infurrowing.

Similar abnormalities in tetrads have been reported in the dv meiotic mutant of maize (Staiger and Cande, 1990) and in plants with faulty meiosis due to hybridity (Brown and Lemmon, 1989) and polyploidy (Juel, 1897; Brown and
In summary, treatment of *Magnolia* microsporocytes with griseofulvin resulted in coenocytes containing abnormal numbers and arrangements of nuclei and led to altered patterns of cytokinesis. Division planes appeared to be defined by the interaction of radial perinuclear systems of microtubules. This evidence is taken as supporting the cytoplasmic domain model for the control of division plane and organization of phragmoplasts.

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References


