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Abnormal cytoskeletal and chromosome distribution in *po*, *ms4* and *ms6;* mutant alleles of *polymitotic* that disrupt the cell cycle progression from meiosis to mitosis in maize

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SUMMARY

The maize cell cycle regulation mutant *polymitotic* (*po*) progresses through abnormal cell cycles, characterized by premature cell divisions without chromosome duplication of the daughter cells produced by meiosis during microsporogenesis and macrosporogenesis. There are three recessive alleles of the *Po* gene; *po, ms4,* and *ms6*. A new method of permeabilizing cells based on freeze-fracture technology was used to study the distribution of microtubules in wild-type and mutant microspores. Here we show that an abnormal distribution of microtubules is correlated with changes in chromosome morphology in a cell cycle-dependent manner in *po, ms4* and *ms6* mutant alleles. After meiosis II, the cell cycle is complete and becomes progressively less synchronous in *po* homozygotes compared with wild-type cells. During microsporogenesis, the distribution of microtubules is abnormal, and chromosome morphology is altered in both *po, ms4* and *ms6* mutants. However, more chromosome fragments or micronuclei associated with mini-spindles are present in *ms6* than *po* and *ms4*. After microspores are released from the tetrads, disruptions in structure and organization of chromosomes and microtubules continues in subsequent abnormal cell cycles. However, these cell cycles are incomplete since phragmoplasts are not formed. During these incomplete cell cycles, abnormal spindles and microtubule arrays are induced and extra microtubule arrays are associated with irregularly distributed chromosome fragments. States corresponding to interphase, prophase, metaphase and anaphase can be recognized in the mutant microspores. Abnormal cell cycles also occur after female meiosis during *ms4* macrospore development. Since only the cell that normally undergoes embryo sac development (the chazal-most cell) undergoes supernumerary divisions this suggests that the *po* phenotype can be characterized as premature haploid divisions rather than repetition of meiosis II.

Key words: meiosis, chromosomes, microtubule, microsporogenesis, maize meiotic mutants, cell cycle

INTRODUCTION

Although the regulation of the cell cycle has been studied in many organisms (Nurse, 1990), relatively little is known about its mechanism of regulation in plants (Feiler and Jacobs, 1990; John et al., 1989, 1990; Colasanti et al., 1991). Maize microsporogenesis has the potential to be a model system for studying cell division and the progression of the cell cycle in higher plants. During maize microsporogenesis, the meiocytes (the pollen mother cells) proceed through a well-defined sequence of meiotic and mitotic cell divisions to yield pollen grains. The availability of a large collection of maize meiotic mutants (Beadle, 1932; Burnham, 1982; Carlson, 1988; Clark, 1940; Coe et al., 1988; Golubovskaya, 1989; Rhoades, 1950; Sheridan, 1982; Staiger and Cande, 1990, 1991) provides an unique opportunity to study perturbations in the mechanisms of cell division and cell cycle regulation. The large meiocytes are superb cytological specimens making possible a detailed analysis of how these mutations affect chromosome morphology and cytoskeleton distribution throughout the cell cycle. Several maize mutants may be defective in cell cycle regulation of meiosis (Coe et al., 1988; Golubovskaya, 1989; Rhoades, 1950). These include *am (ameiotic),* which substitutes a mitotic division for the first meiotic division; *afd (absence of first division),* which has an altered meiosis I; *el (elongate)*, which is involved in controlling meiosis II; and the recessive alleles of *polymitotic* (*po*), which alter the transition from meiosis to the subsequent haploid mitosis without DNA replication. Investigation of these mutants should help to define the mechanism of cell cycle regulation in plants. In particular, analysis of *po* and its alle-

les should help us understand the relationship between DNA synthesis and the rest of the cell cycle.

The mechanism coupling DNA replication to entrance into M phase, and the identification of potential regulatory components involved in this process have been studied in a variety of eukaryotic cells. In the fission yeast mutant *pim* 1 (premature initiation of mitosis), onset of mitosis is uncoupled from the completion of DNA replication, and cells undergo mitotic chromosome condensation and mitotic spindle formation without completion of S phase (Matsumoto and Beach, 1991). A mutant similar to *po* in maize has been identified in *Neurospora*, and it is characterized by cell divisions following meiosis without any accompanying S phase (Raju, 1986). In wild-type *Drosophila* embryos, S phase can be inhibited by injecting aphidicolin. However, chromosomes continue to condense and decondense (Glover et al., 1989). The relationship between DNA replication and cell cycle progression has also been studied in vitro with *Xenopus* egg extracts after similar treatments (Dasso and Newport, 1990). Mitosis can also be uncoupled from the completion of DNA replication in BHK cells by caffeine treatment (Schlegel and Pardee, 1986, 1987).

Analysis of maize cell cycle regulation mutants offers a unique opportunity to study the mechanism coupling DNA replication to cell cycle progression in multicellular eukaryotic systems. Mutant alleles of the maize *polymitotic* gene, *po, ms4 (male sterile 4)* and *ms6 (male sterile 6)*, disrupt S phase during the progression from meiosis to mitosis (Beadle, 1929, 1931, 1932; Albertson and Phillips, 1981; Golubovskaya and Urbach, 1981; Golubovskaya and Khristolyubova, 1985; Golubovskaya, 1989; Liu, unpublished data). The mutant phenotypes were characterized cytologically as premature haploid mitotic cell divisions or reiterations of meiosis II without chromosome replication (Beadle, 1929, 1931; Albertson and Phillips, 1981; West, 1985; D. P. West and R. L. Phillips, personal communication; West and Phillips, 1985). During multiple abnormal cell divisions of the *po* mutant, chromosomal fragmentation occurred (Beadle 1929, 1931). Here, we have analyzed cellular changes in the distribution and morphology of microtubules and chromosomes in *po*, *ms4* and *ms6* cells during abnormal microspore and macrospore cell cycles. The cellular phenotypes we observed suggest that the *Po* gene specifies a protein that is involved in the transition from the meiotic to the haploid mitotic cell cycle.

MATERIALS AND METHODS

Plant material

Maize (*Zea mays* L.) seeds of the wild-type (inbred B73), the mutant strain (stock), *po* and *ms6*, (Maize Genetics Cooperative Stock Center, Urbara, IL), and *ms4* (seeds from the personal colection of I. N. Golubovskaya) were germinated in vermiculite flats for 15-25 days, and then transferred to pot cultures and grown in the greenhouse. After two months of growth, stages of tassel development were identified, and tissue samples were collected according to Staiger and Cande (1990). Allelic tests for *po, ms4* and *ms6* were also carried out in both greenhouse and field conditions by testcrossing *po* with *ms4* and *ms6* (Albertson and Phillips, 1981; Golubovskaya and Urbach, 1981).

Cellular analysis of male microsporogenesis

Chromatin staining with 4′,6-diamidino-2-phenylindole (DAPI) was used to visualize chromatin structure at all stages of microsporogenesis in both mutant and wild-type cells by using fluorescence light microscopy. Tassel samples were collected at different stages and fixed in ethanol:acetic acid (3:1) and transferred to 70% ethanol for cellular analysis (Burnham, 1982). After anthers were dissected on slides, meiocytes or microspores were extruded and incubated with DAPI (Sigma) at 1 μg/ml in 70% ethanol. To analyze different stages of the cell cycle, samples were collected from the same tassel, and 3 anthers from spikelets at a similar developmental stage were dissected. The different stages of the cell cycle in the anthers were based on chromatin morphology and each stage was scored as the percentage of total cells in the anthers. Mitotic cell divisions were also investigated by using root tip meristem tissue of mutant and wild-type plants (Hogan, 1987).

Cellular analysis of female macrosporogenesis

Female meiosis in maize archeosporic cells was studied by using a modified squash/enzyme dissection technique of ovary isolation. Briefly, 2-4 cm young ears were dissected from leaf envelopes and fixed in Chemberlenes fixative (Golubovskaya et al., 1992). After fixation, ears were washed with 95% ethanol and kept in 70% ethanol in a refrigerator until use. Ears were then incubated in 2% pectinase solution for 24 hours at room temperature to digest the cell walls. Then, the ears were treated by cold hydrolysis at room temperature as follows: 1.5 hours in 1 M HCl, 2 hours in 50% HCl, followed by 1 hour in 1 M HCl. The ears were then incubated in Feulgen's reagent for 3 hours to stain all cells, then the ears were washed three times in distilled water. Ear florets were placed on a slide in a drop of glycerol and 45% acetic acid mixed together in a 1:1 ratio. Archeosporic cells (megaspore mother cells) dissected away from the surrounding ovarian tissue, were used to analyze all stages of female meiosis.

Indirect immunofluorescence

Cells were double stained with DAPI and an antibody against tubulin (a monoclonal antibody against sea urchin flagellar tubulin, a generous gift from Dr David Asai, Department of Biology, Purdue University). Anther samples from tassels were collected and fixed in 4% paraformaldehyde in PIPES/EGTA medium (Tiwari and Polito, 1990; Staiger and Cande, 1990) for at least 40 minutes. More than 50 anthers at similar stages from the same tassel were dissected in Petri dishes. Fixed meiocytes or microspores were transferred to a round coverslip, and another coverslip was put on top to make a sandwich for freeze-fracture. The cells in this coverslip sandwich were quickly frozen in liquid propane and liquid nitrogen for 20-30 seconds, and then fractured by forcing the coverslips apart. Freeze-fractured cells were collected in 1 ml centrifuge tubes and centrifuged at low speed (1,000 rpm) at room temperature for 10 minutes to pool the sample for washing with PBS (phosphate-buffered saline) buffer at least three times. Cells were then incubated in PBS plus 0.5% Triton X-100 for 30 minutes, and then incubated in primary antibody (1:200 or 500 dilution) for 1-2 hours at 37°C or overnight at room temperature. Cells were then washed with PBS at least 3 times, and incubated in 0.1 mg/ml fluorescein-conjugated goat anti-mouse IgG (Cappel laboratories Malvern, PA) for 1-2 hours at 37°C, followed by three washes with PBS buffer. DAPI (1 μg/ml) was added in the final wash, the cells were then pipetted onto slides, and coverslips were mounted with 90% glycerol containing 100 mg/ml of antioxidant 1,4-diazobicyclo-(2,2,2)-octane (Ernest F. Fullam,

Schenectady, NY). As a control, samples were processed as described above except for omission of primary antibody.

Cells in microsporogenesis were examined on a Zeiss Axiophot microscope in the NSF Plant Development Center, University of California, Berkeley, and images were recorded on Kodax Tmax film (400 ASA) and developed in Kodak D-76. Cells in macrosporogenesis were examined using a Biolar light microscope and images were taken with a MFN II Micro Camera.

RESULTS

Chromosome morphology and microtubule distribution during microsporogenesis in wild-type cells

Microtubule distribution during microsporogenesis has been described in wild-type meiocytes after enzymatic digestion of their cell walls to make the cells permeable to antibodies (Staiger and Cande, 1990). However, these enzyme mixtures cannot digest the microspore cell wall, limiting our investigation of changes in cytoskeletal arrangement during development to events before tetrad formation (Staiger and Cande, 1990, 1991). In this study, a freeze-fracture method was used to rupture both meiocyte and microspore cell walls and allowed us to circumvent this obstacle. Meiotic spindle structure is similar in meiocytes permeabilized by freeze-fracture (Fig. 1B) or enzymatic digestion (Staiger and Cande, 1990) and chromosome morphology is normal (Fig. 1A), thus demonstrating that this new method of permeabilizing cells yields similar results to that used previously. In another example, four interphase nuclei from tetrads (Fig. 1C) show an even distribution of microtubule arrays extending from the nuclei to the cell periphery (Fig. 1D).

As the callose wall around the tetrads disintegrated, microspores are released. The transition from meiosis to pollen mitosis took 7 to 10 days. During this period, microspore nuclei are in interphase (data not shown) and pollen walls were formed. The interphase microtubule arrays are also maintained. As a result of the first pollen mitosis, vegetative and generative nuclei are produced (data not shown). Normal healthy pollen grains are formed.

Chromosome morphology and microtubule distribution in *po* meiocytes and microspores

Abnormal progression through the meiotic cell cycle is first shown by the presence of asynchronous meiocytes in late stages of meiosis II. The *po* meiocyte cells undergo a normal anaphase II and telophase II as shown by the presence of apparently normal spindles and phragmoplasts (data not shown). However, even within one tetrad, asynchronous cell divisions are observed in late stages of meiosis II (Fig. 2A and B). In Fig. 2A, two of the tetrads have not yet completed cytokinesis, while the other two have entered the equivalent of a post-tetrad interphase. The interphase nuclear morphology is abnormal in these cells. The chromatin is more diffuse than that in wild-type cells at a similar stage (compare to Fig. 1C). In Fig. 2B, two tetrad cells show abnormal interphase nuclear microtubule arrays. However, the other two cells are separated by a phragmoplast. In wild-type cells all four cells would be at exactly the same stage in the cell cycle.

The abnormal divisions occurred immediately after meiosis II and are asynchronous within the anther and even within one tetrad (data not shown). At this early stage of post-tetrad development, the tetrad cells were embedded in an amorphous callose wall. This abnormal cell cycle is characterized by abnormal cell divisions without chromosome duplication (Fig. 2C). Although the chromosomes take on a compact rod shape they are not doubled (Fig. 2C). As a consequence of this abnormal chromosomal segregation (Fig. 2D), some microspore cells have many chromosome fragments of disparate and small size (Fig. 3C and 3E). The chromosome fragmentation and the reduction in chromosome number in these abnormal cell divisions is consistent with Beadle's original observations concerning the absence of chromosome replication (Beadle 1928, 1931).

After tetrad formation, the *po* cells immediately form spindles (Fig. 2E). The microtubule arrays in mutant cells are abnormal. Microspindles (Fig. 2E) are associated with

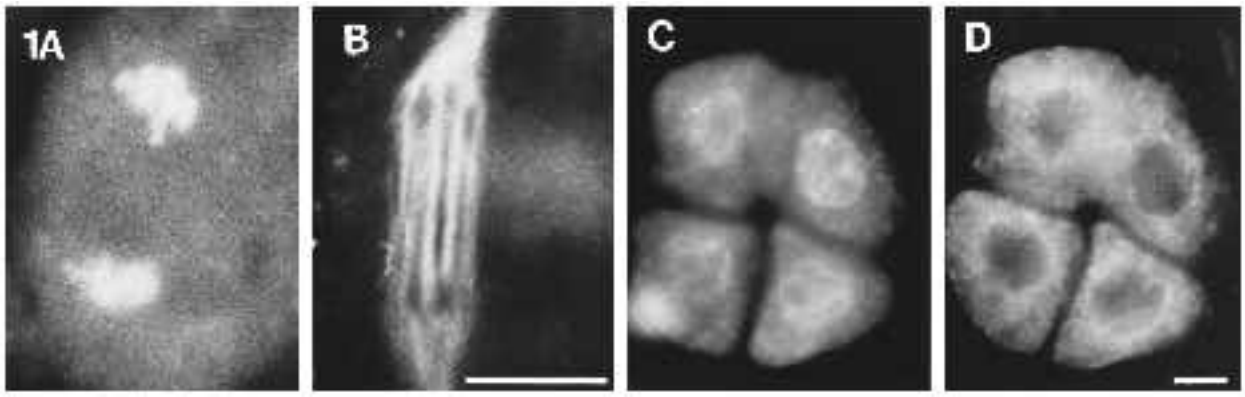

Fig. 1. Microtubule distribution and chromosome morphology in freeze-fractured wild-type meiocytes as visualized by epi-fluorescence optics using an antibody against tubulin and a DAPI stain for chromatin. (A-B) Focused meiotic spindles (B) and normally segregating chromosomes (A) are observed in anaphase I of the same meiocyte. (C-D) Interphase microtubule arrays (D) are associated with each nucleus (C) of the tetrad. Bars, 5 μm.

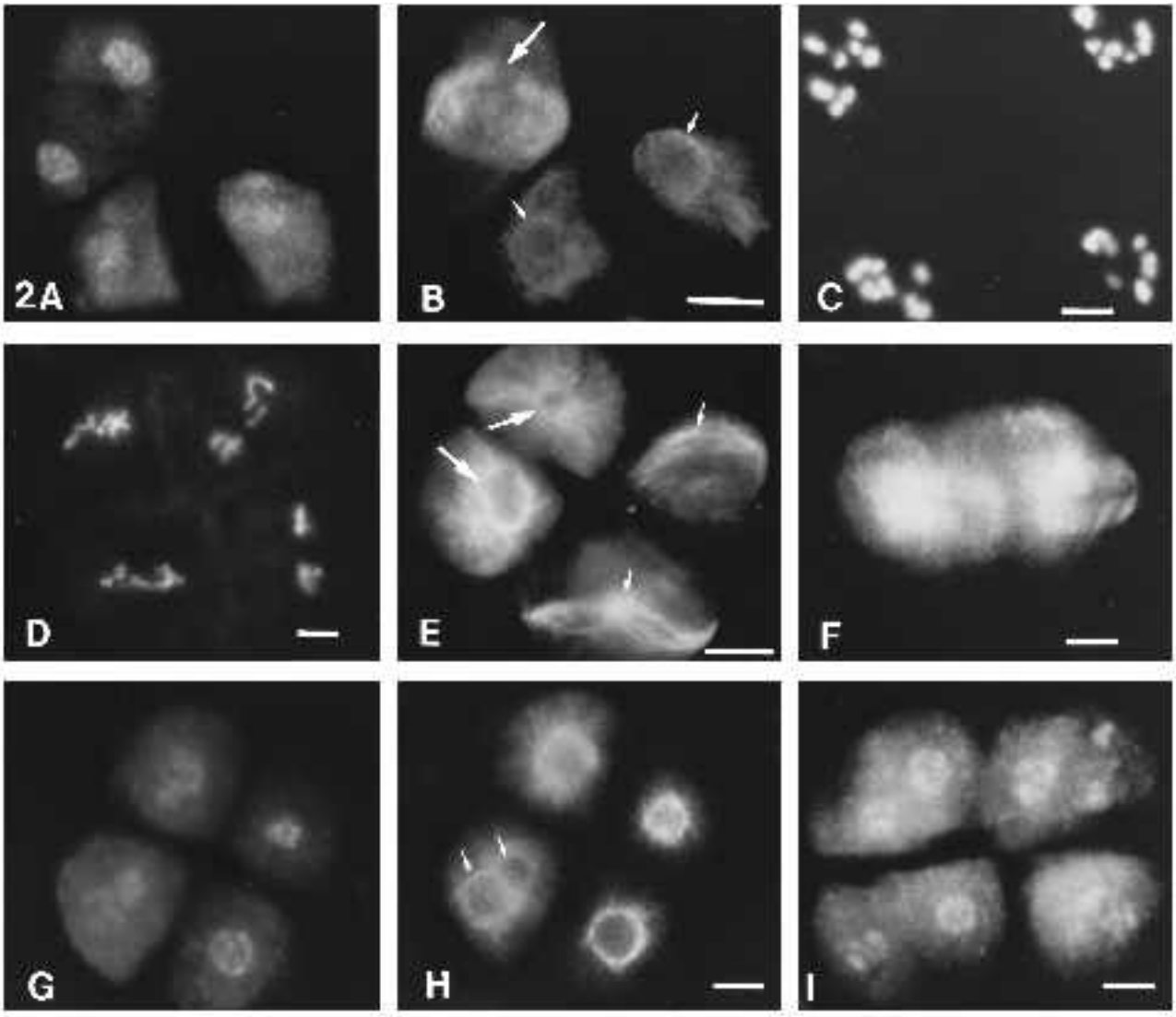

Fig. 2. Abnormal microtubule arrays and chromatin morphology in *po* tetrads. (A and B) Double-stained *po* meiocytes (stained as described in Fig. 1) show abnormal chromatin morphology (A), and phragmoplast (B, large arrow) in telophase II and unevenly distributed nuclear associated microtubules (B, small arrows). This is an example of the initiation of asynchronous cell divisions after telophase II. (C and D) As visualized using DAPI, *po* meiocytes display unduplicated chromosomes (C, n=10) and undergo segregation of chromosomes (D) following abnormal cell divisions. (E and F) In two tetrad cells, there are abnormal interphase microtubule arrays (E, large arrows), each with irregularly distributed microtubules. In the other two cells of the same tetrad, abnormally focused meiotic-like spindles are present, each with a different morphology (E, small arrows). (F) Four overlapping phragmoplasts form following abnormal cell divisions of the tetrad. (G and H) A double-stained tetrad shows irregularly distributed interphase microtubule arrays (H) associated with abnormally condensed chromatin (G). Within one of the tetrad cells two perinuclear microtubule arrays surrounding each of the condensed chromatin groups (H, arrows) can be seen. (I) DAPI staining of chromatin in *po* meiocytes shows more than 8 interphase nuclei as a result of abnormal cell divisions of the tetrad. Bars, 5 μm.

randomly segregating chromosome fragments (data not shown). In the other two cells of the same tetrad, microtubules extend unevenly from the nuclear envelope (Fig. 2E). Binucleate microtubule arrays were also seen (Fig. 2H) in association with abnormally condensed and unevenly distributed chromosomes (Fig. 2G). Phragmoplasts are also formed (Fig. 2F). As a consequence of these abnormal divisions, more than 8 meiotic-like products are formed before the cells are released from the callose walls (Fig. 2I).

Later stages of *po* microspore development are marked by a breakdown of the callose wall and release of the microspores. At this stage of development, irregular distributions of chromatin with morphologies corresponding to interphase, prophase, metaphase and anaphase are observed in microspores from adjacent anthers (Fig. 6), showing that these abnormal multiple cell cycles are unsynchronized and disordered. Poorly organized spindles are also observed in many cells. At a metaphase-like stage, spindle poles are tightly focused with many abnormal chromosome fragments aligned on the metaphase plate (Fig. 3A and B). However, in the same cell, chromosomal frag-

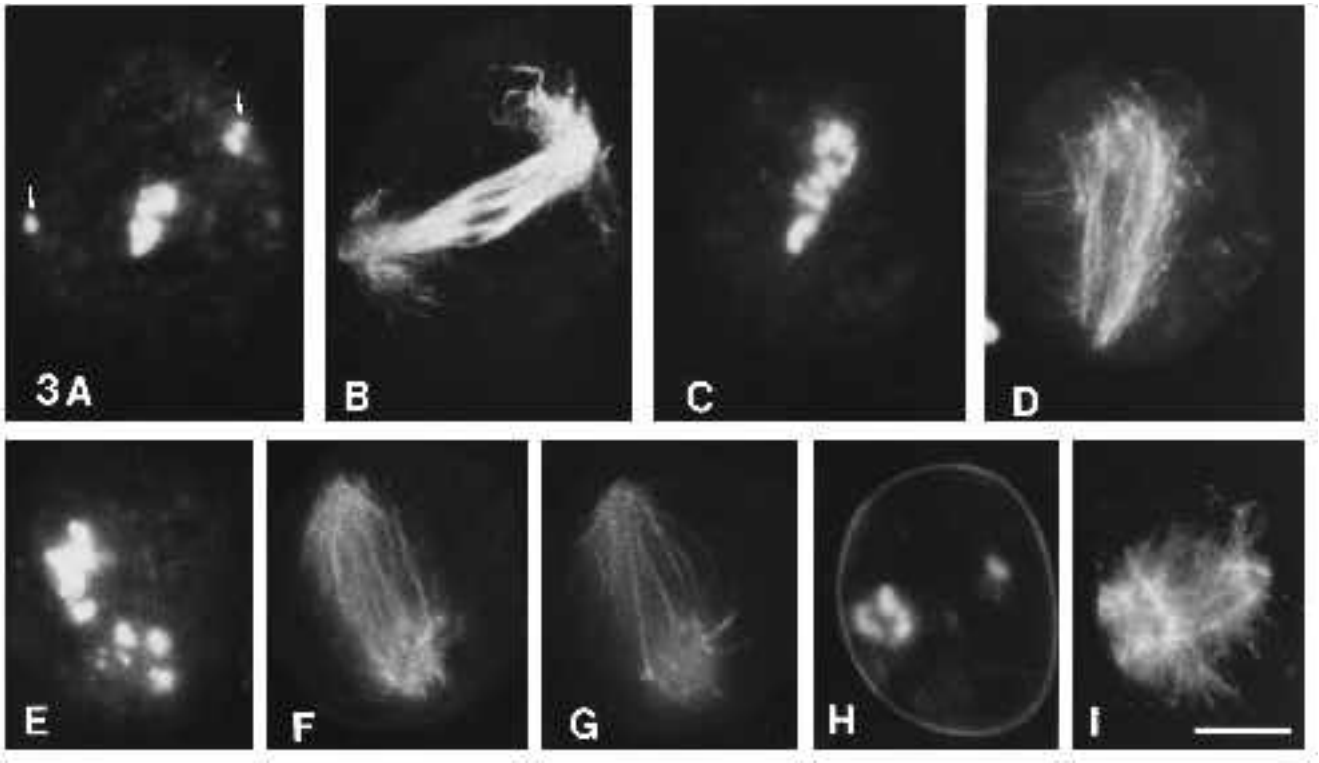

Fig. 3. Abnormal spindle microtubules and chromatin morphology in *po* microspores (stained as described in Fig. 1). (A-B) Abnormal spindle microtubule arrays (B) are associated with chromosomes at the metaphase plate (A), and two lagging chromosomes (A, arrows) are at the spindle poles with their own microtubule array. (C and D) Irregularly distributed chromosomal fragments (C) are aligned along poorly focused spindle microtubule arrays (D). (E-G) Spindle microtubules (F) are associated with irregularly distributed chromosome fragments in an anaphase-like stage of the same microspore (E). In a micrograph taken at a different focal plane of the same cell, a bundle of microtubules (G) appear to run from one pole to the other through these chromosome fragments (E). (H and I) A microtubule array (I) is associated with two groups of chromosome fragments (H) that are unevenly distributed in the microspore cell. The staining for microtubules is very pronounced surrounding these chromosome fragments. Bar, 5 μm.

ments (Fig. 3A arrows) at each pole appear to organize their own spindles (Fig. 3B). Spindles can be ragged and poorly organized and chromosome fragments are randomly aligned on them (Fig. 3C and D) instead of aligned on a metaphase plate. In another example, spindle microtubules are broadly focused and associated with variable size chromosome fragments, which appear to be undergoing an anaphase-like segregation (Fig. 3E,F and G). A bundle of microtubules (Fig. 3G) runs from one pole to the other through these chromosome fragments (Fig. 3F). Spindles with abnormally divergent poles are also observed (data not shown). Furthermore, microtubule arrays are also associated with segregated groups of chromosome fragments. For example, multiple aster-like microtubule arrays radiate from chromosome fragments in opposite regions of the same cell (Fig. 3H and I). However, phragmoplasts are never observed after microspores are released from the abnormal tetrads, suggesting that these cell cycles are incomplete.

In subsequent abnormal cell cycles, perinuclear and cytoplasmic microtubule arrays are associated not only with the interphase nucleus (Fig. 4A and B), but also with condensed late prophase-like chromosomes (Fig. 4C and D). Normally, perinuclear microtubule arrays would break down at this stage (Staiger and Cande, 1990). As a consequence of these incomplete cell cycles without cytokinesis, two interphase nuclear microtubule arrays corresponding to two nuclei and a micronucleus occur in the same cell (Fig. 4E and F). Many microspores containing more than two micronuclei are produced (data not shown). Abnormal cell division activity persists for about one week until chromatin and cytoplasm have disintegrated.

Chromosome morphology and microtubule distribution in *ms4* and *ms6* meiocytes and microspores

Allelic tests have confirmed that both *ms4* and *ms6* are *po* alleles (Golubovskaya and Urbach, 1981; Albertson and Phillips, 1981; data not shown). Similar chromosome and microtubule defects are observed in tetrads of *po*, *ms4* and *ms6* before the microspores are released (Fig. 5 and 6). However, many more chromosome fragments with associated mini-spindles are present in the tetrads of *ms6* (Fig. 5A,B and C). The *ms6* microspores undergo more abnormal cell division cyles than do *po* and *ms4* microspores (Fig. 5D). As a consequence, more microspores are released and there are fewer chromosome fragments or micronuclei per cell in *ms6* than *po* and *ms4*. (Fig. 5E). Thick microspore walls develop after microspores of variable size are released from the tetrads of *ms6* (Fig. 5E).

Abnormal cell divisions in female meiosis of *ms4* macrospores

Female meiosis in wild-type cells has been described in isolated macrospores after the cells were dissected from the ear by a modified squash technique that relies on enzymatic digestion and cold hydrolysis of the cell wall to permit subsequent tissue dissection (Golubovskaya et al., 1992). Although this technique is incompatible with indirect immunofluoresence, chromosomes and phragmoplasts are visible after Feulgen staining. Female meiosis in *ms4* macrospores was analyzed using the same technique. During normal development the large archeosporic cell has an elongated shape (Golubovskaya et al., 1992). The metaphase I spindle forms parallel to the long axis of the cell and the subsequent dyads are different in shape (see also Fig. 7a). Normally the lower (chazal-most) cell enters the second meiotic division before the upper (or micropylar-most) cell (see also Fig. 7b). Often the upper cell never completed meiosis II but degenerated. In any case the lower cell always completed meiosis II and subsequently became the initial cell of the embryo sac.

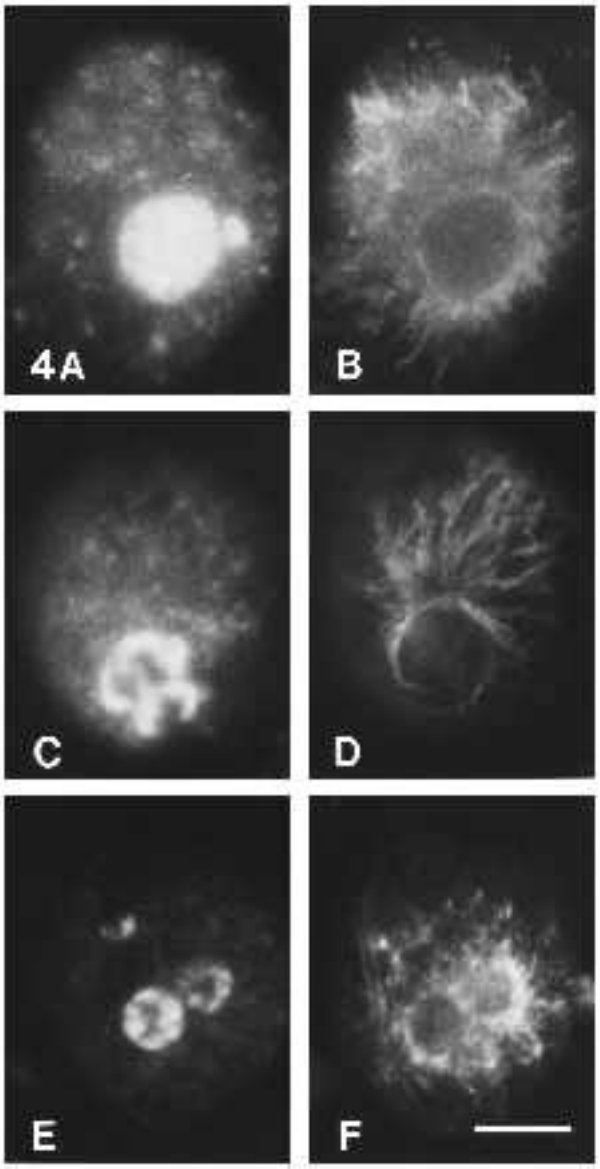

Fig. 4. Abnormal interphase microtubule arrays and chromatin morphology in *po* microspores (stained as described in Fig. 1). (A and B) A microtubule array (B) is associated with the interphase nucleus (A) in one microspore. (C and D) An abnormal nuclear microtubule array (D) is associated with condensed prophase chromosomes (C) in another microspore. (E and F) Interphase microtubule arrays (F) are associated with two nuclei (E) and a micronucleus in the same microspore cell. Bar, 5 μm.

During *ms4* female meiosis, the cell cycle proceeded normally from meiosis I to the end of meiosis II. In telophase I of the dyad stage (Fig. 7A), a phragmaplast was observed between the nucleus of the apical micropilar cell and bottom halazal cell. In meiosis II, the micropilar cell was in prophase and the halazal cell entered telophase (Fig. 7B) as in wild-type plants (Golubovskaya et al., 1992). After this stage, macrosporogenesis was disrupted. Chromosomes in the halazal cell condensed and proceeded to divide abnormally immediately after telophase II (Fig. 7C-E). However, this abnormal division cycle only occured in the bottom daughter cell (Fig. 7D and E), and the top cell remained in an interphase state (Fig. 7E, arrow). Similar abnormal female postmeiotic cell divisions have also been described for the other *po* alleles (West, 1985; Golubovskaya, unpublished data).

DISCUSSION

Overview

Our results suggest that *po*, *ms4* and *ms6* alter the normal transition from meiosis to mitosis during microsporogenesis and macrosporogenesis. The first sign of abnormality is asynchronous divisions associated with meiosis II in microspores. As described previously, we confirmed that *po*, *ms4*, *ms6* are alleles and that chromosome morphology and behavior is altered in *po* terads (Beadle, 1931; West, 1985), *ms4* tetrads (Golubovskaya and Urbach, 1981) and *ms6* tetrads (Albertson and Phillips, 1981). Using a new freeze-fracture method to permeabilize microspore cells, we show that an abnormal distribution of microtubules is correlated with these chromosome alterations in a cell cycle-dependent manner. After microspores are released from the tetrads, the cell cycle-dependent disruption in structure and organization of chromosomes and microtubules continues, but no phragmoplasts are present, suggesting that the cell cycle is further reduced. During these incomplete cell cycles, abnormal spindles and multinucleate microtubule arrays form, but structural association of chromosomes and microtubules is further altered. Although it is difficult to determine the number of supernumerary divisions in this mutant, Beadle (1931) suggested that there were an extra 5-6 abnormal cell cycles, sometimes producing microspore cells with as few as one chromosome.

Cellular analysis of *ms4* female macrosporogenesis showed similar defects in the transition from meiosis to mitosis during male microsprogenesis in *po* plants (West, 1985). However, during *po* macrosporogenesis this abnormality appeared to be developmentally regulated. That is, the only post-meiotic cell that went through additional cell division cycles was the cell that would normally develop

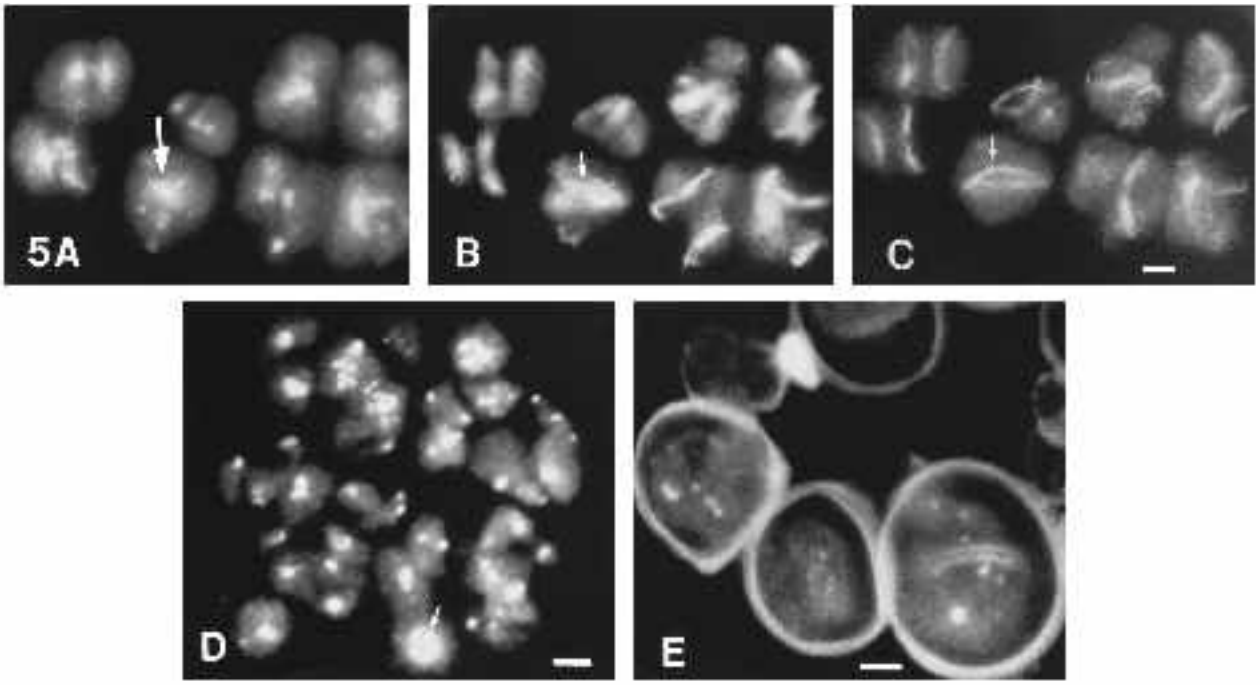

Fig. 5. Microtubule arrays and chromatin morphology in *ms6* tetrad and microspores after abnormal cell divisions. (A-C) In the same *ms6* meiocytes, many abnormal mini-spindles (B and C, arrows in the same cell at different depth of focus) are associated with condensed chromatin (A, large arrow) following abnormal tetrad cell divisions. (D) There are about 30 abnormal small meiocytes with small chromosome fragments or micronuclei, a feature of the mutation that results from supernumerary divisions of the tetrad. The nuclei in the meiocytes are much smaller than in the tapetal cells (D, arrow). (E) In *ms6*, more microspores are produced than in *po*, they are smaller and contain chromosome fragments and micronuclei inside thick cell walls. Bars, 5 μm.

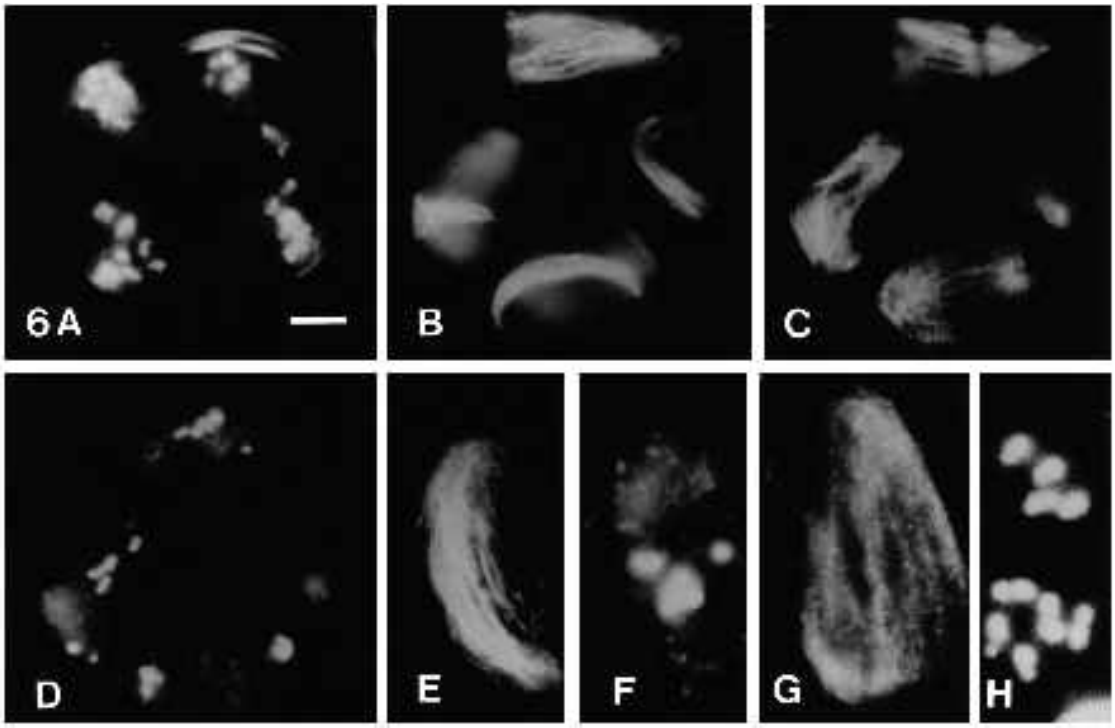

Fig. 6. Microtubule arrays and chromatin morphology in *ms4* tetrad and microspores after abnormal cell divisions. In *ms4*, meiocytes display condensed chromosomes (A) and undergo random segregation (D) following abnormal cell divisions. There are abnormal spindle microtubule arrays (B and C in the same cell at different depth of focus), each associated with these irregularly distributed chromosomes. Metaphase-like spindle arrays (E) are associated with a few chromosome fragments (F) at this stage. Broad focused spindle microtubule arrays (G) are associated with two groups of chromosome fragments (H) that are unevenly distributed in the microspore cell in an anaphase-like stage. Bar, 5 μm.

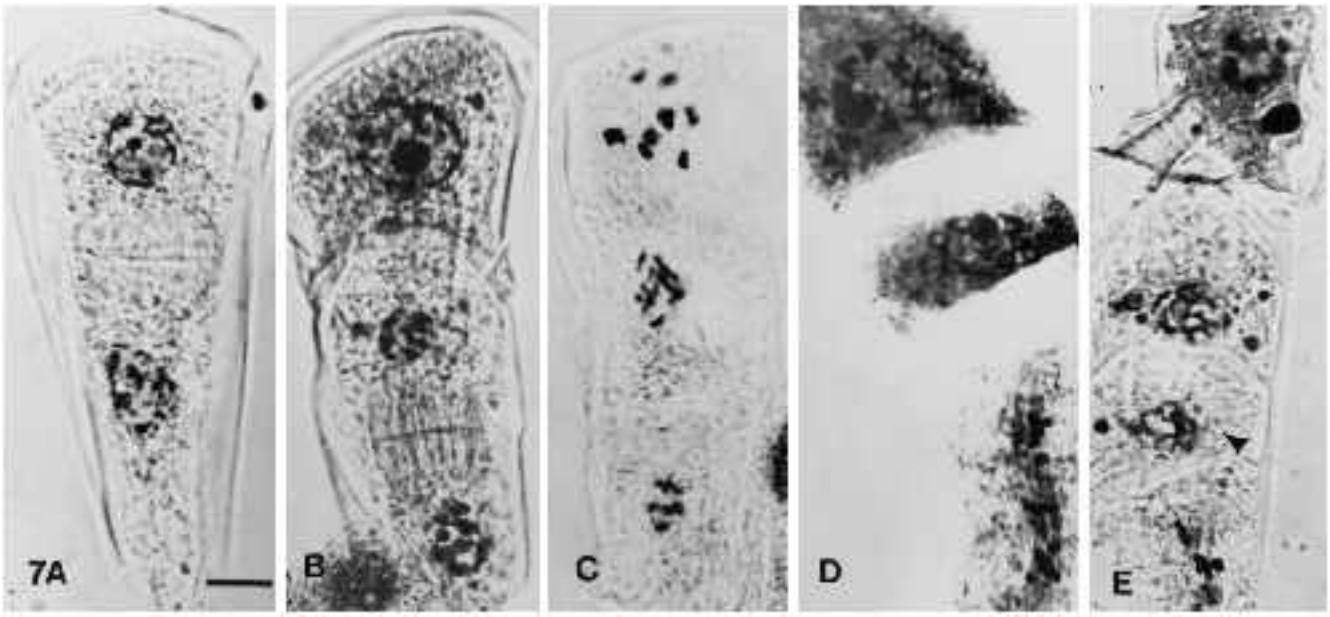

Fig. 7. Abnormal cell divisions in female meiosis of ms4 macrosporogenesis. Female meioses of ms4 macrospores have been investigated by using a squash technique of ovary isolation as described in Material and Methods. Cells were Feulgen stained and viewed with bright field optics. (A) In telophase I, a phragmaplast was seen between the nuclei of the apical micropilar cell and the bottom halazal cell. (B) In meiosis II, the micropilar cell was in prophase and the halazal cell entered telophase II as in wild-type plants (Golubovskaya et al., 1992). Chromosomes in the halazal cell condensed and entered into an abnormal cell division immediately after telophase II (C and D). However, this abnormal division only continued in the bottom daughter cell of the divided halazal cell (D and E), and the top daughter cell remained in interphase (E, arrowhead). Bar, 5 μm.

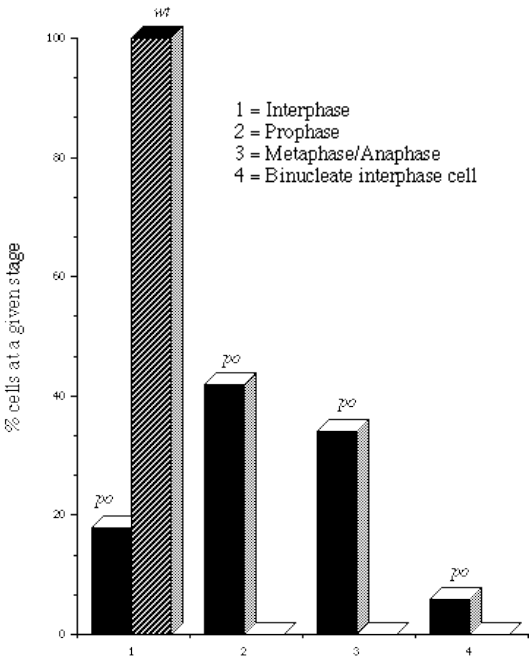

Fig. 8. Cell cycle stages present in a population of *po* vs wild-type microspores in a similar developmental stage. For the data presented here, anthers from a spikelet at a similar developmental stage (immediately post-meiosis) were collected from a *po* and wild-type plant. The cells from these anthers were pooled together and stages in the cell cycle were determined based on chromosome morphology. Each stage is represented as percentage of the total cells observed.

as the embryo sac. This result is consistent with the suggestion that *po* is a gene that is involved in regulating the postmeiotic haploid cell cycles rather than the exit from meiosis.

More chromosome fragments or micronuclei associated with mini-spindles were observed in *ms6* than in *po* and *ms4* after tetrad formation. There are several possible explanations for this result: either *ms6* is a stronger allele in disrupting the cell cycle, or, cell divisions in *ms6* occur at a faster rate at the same stage than in *po* and *ms4*. Alternatively, microspores of *po* and *ms4* may be released earlier than *ms6*. Some of these differences could be due to the genetic background of the different inbred lines. We are interested in studying the role and sequence of events in *po*, *ms6* and *ms4* in the transition from meiosis to mitosis by combined cell biology and genetic approaches. We are now in the process of crossing these alleles into the same inbred background to make a more accurate comparison of the function of each allele.

Abnormal microtubule arrays and chromosome morphology are associated with the abnormal cell divisions of *po*, *ms4* and *ms6* mutants

We observe that the microtubule distribution pattern is disturbed during the abnormal *po* cell cycles and that randomly scattered chromosome fragments or micronuclei are often associated with irregularly shaped spindles and nuclear envelope-associated microtubules. Some of these abnormalities may be a direct consequence of having unreplicated or prematurely condensed chromosomes

rather than abnormal centrosome activity. First, many of the microtubule arrays are associated with clusters of ill-defined chromosomes. Second, the spindles that are formed during the *po* cell cycles are grossly disturbed. In one example, spindle poles are less focused or contain arrays of microtubules running from one pole to the other pole, and chromosome fragments have difficulty in aligning on the metaphase plate. Similar spindle abnormalities are also seen in a desynaptic mutant (*dsy2*) when a large number of univalents are present (R. K. Dawe, personal communication). However, in *po*, *ms4* and *ms6*, premature chromosome condensation may generate these abnormalities. The presence of individual extra chromosome fragments each associated with its own microtubule array suggest that chromatin components play an active role in organizing and stabilizing microtubule arrays. This conclusion is consistent with studies of meiotic and mitotic spindle formation in a variety of organisms (Gard, 1992; Sawin and Mitchison, 1991; Theurkauf and Hawley, 1992; Leslie 1992). The kinetochore is a specialized structure attaching the chromosome to the spindle and is the obvious candidate for organize microtubule arrays. During the incomplete cell cycles in *po* tetrads it is unlikely that the kinetochores are replicated and may be improperly positioned on the condensed chromatin (see Brinkley et al., 1988). In desynaptic mutants such as *dsy2*, univalent chromosomes during meiosis I will have kinetochores that are asymmetrically positioned in the centromere. It is not surprising then that these two classes of abnormal chromosomes, although generated by very different mutations, should have similar effects on spindle organization if the kinetochore function and symmetry is involved. However, Brinkley et al. (1988) have shown that chromosome replication is not a prerequisite for alignment and segregation of kinetochores in Chinese hamster ovary cells. In this cell line, unreplicated kinetochores without any attached chromatin can interact with microtubules and complete all mitotic movements.

Possible models for defects in cell cycle regulation of *po*, *ms4*, and *ms6* mutants

The cellular phenotypes we observed suggest that the *Po* gene specifies a protein that is required for the transition from the meiotic to the haploid mitotic cell cycle. There are several possible models that can explain the defects in cell cycle regulation we observed in the *po*, *ms4*, and *ms6* mutants. First, it is possible that meiosis continues without chromosome duplication. The abnormal cell cycle would be due to a repetition of meiosis II as suggested by West and Philips (West and Phillips, 1985). Thus, in this model the defect is due to an inability of the mutant cell to determine whether it has gone through meiosis II and it repeats it over again. Alternatively, premature pollen mitosis may be induced without chromosome duplication, an event which normally occurs one week later in development. In this model the defect is in the regulation of the mitotic cell cycle and the microspore undergoes M phase without an accompanying S phase. Cellular analysis of abnormal cell divisions during *ms4* female meiosis support the second model. In the female, the abnormal cell cycle only occurs in the one tetrad macrospore, which is scheduled to undergo haploid cell divisions and does not occur in the other cells, which normally degenerate rather than divide further. Thus, the abnormal *po* cell cycle is correlated with the possibility of future haploid divisions rather than the completion of meiosis II.

The mechanism of coupling of DNA replication and mitosis have been investigated in many eukayotic cell cycles including that of *Aspergillus nidulans* (Osmani et al., 1988) and *Drosophila* (Raff and Glover, 1988). Experimental manipulation of the *Drosophila* embryonic mitotic cycle suggest that DNA replication was not required for cycles of chromatin condensation/decondensation and nuclear envelope breakdown/reformation (Raff and Glover, 1988). In this case, mitosis continues even when DNA synthesis was inhibited by aphidicolin. Based on these experiments, Raff and Glover (1988) suggested that a cell cycle oscillator operates independently of the nucleus, and an oscillating cytoplasmic signal could drive the nuclear cycle.

Regulation of chromatin condensation may be a key step in controlling cell cycle-dependent DNA replication. The RCC1 gene has been implicated in regulation of chromatin condensation in human cells. The RCC1 gene product is localized in the nucleus and binds to DNA (Ohtsubo et al., 1989). It complements the temperature-sensitive (ts) BN2 mutation, a ts mutant in the baby hamster kidney (BHK21) cell line, which induces premature chromosome condensation at the restrictive temperature (Ohtsubo et al., 1987). Nishimoto et al. (1978, 1981) suggest that following premature chromatin condensation, DNA replication ceases. This is similar to *po* in that premature chromatin condensation occurs without DNA replication. It is not obvious whether a defect in the RCC1 gene product would result in the maintenance of nuclear and microtubule cycles without chromosome replication, as is observed in *po*. Research on bimE7, a temperature-sensitive cell cycle mutation in *Aspergillus nidulans*, suggests that a negative cell cycle control gene plays an important role in the normal coupling of DNA replication and mitosis (Osmani et al., 1988). The bim E7 mutation causes premature spindle formation and chromatin condensation in S or G_2 cells. This mutation can override normal cell cycle regulation and lead to premature mitosis before completion of DNA synthesis. It is possible that *po* encodes a gene product that is a functional analog of bim E7 during maize microsporogenesis. To confirm this possibility, we plan to use a molecular genetic approach to identify the *Po* gene product. The analysis of the *po* mutant and its alleles at the molecular and genetic level should provide a further insight into the mechanism of meiotic cell cycle regulation and address how it may differ from mitotic cell cycle regulation.

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REFERENCES

Albertson, M. C. and Philips, R. L. (1981). Developmental cytology of 13 genetic male sterile loci in maize. *Can. J. Genet. Cytol.* **23**, 195-208.

Beadle, G. W. (1929). A gene for supernumerary mitoses during spore development in *Zea mays.Science* **50**, 406-407.

Beadle, G. W. (1931). A gene in maize for supernumerary cell divisions following meiosis. *Cornell Univ. Agr. Exp. Sta. Mem.* **135**, 1-12.

Beadle, G. W. (1932). Genes in maize for pollen sterility. *Genetics* **17**, 413-431.

Brinkley, B. R., Zinkowski, R. P., Mollon, W. L., Davis, F. M., Pisegna, M. A. Pershouse, M. and Rao, P. N. (1988). Movement and segregation of kinetochores experimentally detached from mammalian chromosomes. *Nature* **336**, 251-4.

Burnham, C. R. (1982). Details of the smear technique for studying chromosomes in maize. In *Maize for Biological Research* (ed. W. F. Sheridan), pp. 107-118. University Press, Grand Forks, ND, USA.

Carlson, W. R. (1988). The cytogenetics of corn. In *Corn and Corn Improvement.* Agronomy Series, no. 18 (ed. G. F. Sprague and J. W. Dudley), pp. 259-343. American Society of Agronomy, Madison, WI, USA.

Clark, F. J. (1940). Cytogenetic studies of divergent meiotic spindle formation in *Zea mays. Amer. J. Bot.* **27**, 547-559.

Coe, E. H. Jr, Neuffer, M. G. and Hoisington, D. A. (1988). The genetics of corn. In *Corn and Corn Improvement.* Agronomy Series, no. 18 (ed. G. F. Sprague and J. W. Dudley), pp. 81-259. American Society of Agronomy, Madison, WI, USA.

Colasanti, J., Tyers, M. and Sundaresan, V. (1991). Isolation and characterization of cDNA clones encoding a functional $p34^{cdc2}$ homologue from Zea mays. *Proc. Nat. Acad. Sci. USA* **88**, 3377-81.

Dasso, M. and Newport, J. W. (1990). Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in Xenopus. *Cell* **61**, 811-23.

Dasso, M., Nishitani, H., Kornbluth, S., Nishimoto, T. and Newport, J. W. (1992). RCC1, a regulator of mitosis, is essential for DNA replication. *Mol. Cell. Biol.* **12**, 3337-45.

Feiler, H. S. and Jacobs, T. W. (1990). Cell division in higher plants: a cdc2 gene, its 34-kDa product, and histone H1 kinase activity in pea. *Proc. Nat. Acad. Sci. USA.* **87**, 5397-401.

Feiler, H. S. and Jacobs, T. W. (1991). Cloning of the pea cdc2 homologue by efficient immunological screening of PCR products. *Pl. Mol. Biol.* **17**, 321-33.

Gard, D. L. (1992). Microtubule organization during maturation of Xenopus oocytes: assembly and rotation of the meiotic spindles. *Dev. Biol.* **151**, 516-30.

Glover, D. M., Alphey, L., Axton, J. M., Cheshire, A., Dalby, B., Freeman, M., Girdham, C., Gonzalez, C., Karess, R. E. and Leibowitz, M. H. (1989). Mitosis in *Drosophila* development. *J. Cell Sci. Suppl.* **12**, 277-91.

Golubovskaya, I. N. (1989). Meiosis in maize: *mei* genes and conception of genetic control of meiosis. *Advan. Genet.* **26**, 149-192.

Golubovskaya, I. N., Avalkina, N. A. and Sheridan, W. F. (1992). Effects of several meiotic mutations on female meiosis in maize. *Dev. Genet.* **13**, 411-424.

Golubovskaya, I. N. and Khristolyubova, N. B. (1985). The cytogenetic evidence of the gene control of meiosis: maize meiosis and Mei-genes. In *Plant Genetics*. UCLA Symposium on Molecular and Cellular Biology, vol. 35 (ed. M. Freeling), pp. 723-738. A. R. Liss, New York.

Golubovskaya, I. N. and Urbach, V. G. (1981). Study of the allelism of meiotic mutations with phenotypically similar disturbances at meiosis. *Sov. Genet.* (Engl. Transl.) **17**, 1975-1982.

Gonzalez, C., Saunders, R. D., Casal, J., Molina, I., Carmena, M., Ripoll, P. and Glover, D. M. (1990). Mutations at the asp locus of *Drosophila* lead to multiple free centrosomes in syncytial embryos, but restrict centrosome duplication in larval neuroblasts. *J. Cell Sci.* **96**, 605-16.

Hogan, C. J. (1987). Microtubule patterns during meiosis in two higher plant species. *Protoplasma* **138**, 126-136.

John, P. C., Sek, F. J. and Lee, M. G. (1989). A homolog of the cell cycle control protein $p34^{cdc2}$ participates in the division cycle of *Chlamydomonas*, and a similar protein is detectable in higher plants and remote taxa. *Pl. Cell* **1**, 1185-93.

John, P. C., Sek, F. J., Carmichael, J. P. and McCurdy, D. W. (1990). $p34^{cdc2}$ homologue level, cell division, phytohormone responsiveness and cell differentiation in wheat leaves. *J. Cell Sci.* **97**, 627-30.

Leslie, R. J. (1992). Chromosomes attain a metaphase position on half-spindles in the absence of an opposing spindle pole. *J. Cell Sci.* **103**, 125-30.

Matsumoto, T. and Beach, D. (1991). Premature initiation of mitosis in yeast lacking RCC1 or an interacting GTPase. *Cell* **66**, 347-60.

Nishimoto, T., Eilen, E. and Basilico, C. (1978). Premature chromosome condensation in a ts DNA^- mutant of BHK cells. *Cell* **15**, 475-483.

Nishimoto, T., Ishida, R., Ajiro, K., Yamamoto, S. and Takahashi, T. (1981). The synthesis of protein(s) for chromosome condensation may be regulated by a post-transcriptional mechanism. *J. Cell. Physiol.* **109**, 299-308.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. *Nature* **344**, 503-508.

Ohtsubo, M., Kai, R., Furuno, N., Sekiguchi, T., Sekiguchi, M., Hayashida, H., Kuma, K., Miyata, T., Fukushige, S., Murotsu, T., Matsubara, K. and Nishimoto, T. (1987). Isolation and characterization of the active cDNA of the human cell cycle gene (RCC1) involved in the regulation of onset of chromosome condensation. *Genes Dev.* **1**, 585-593.

Ohtsubo, M., Okazaki, H. and Nishimoto, T. (1989). The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. *J. Cell Biol.* **109**, 1389-97.

Osmani, S. A., Engle, D. B., Doonan, J. H., and Morris, N. R. (1988). Spindle formation and chromatin condensation in cells blocked at interphase by mutation of a negative cell cycle control gene. *Cell* **52**, 241-51.

Raff, J. W. and Glover, D. M. (1988). Nuclear and cytoplasmic mitotic cycles continue in Drosophila embryos in which DNA synthesis is inhibited with aphidicolin. *J. Cell Biol.* **107**, 2009-19.

Raju, N. B. (1986). Postmeiotic mitoses without chromosome replication in a mutagen-sensitive *Neurospora* mutant. *Exp. Mycol.* **10**, 243-251.

Rhoades, M. M. (1950). Meiosis in maize. *J. Hered.* **41**, 58-67.

Sawin, K. E. and Mitchison, T. J. (1991). Mitotic spindle assembly by two different pathways in vitro. *J. Cell Biol.* **112**, 925-40.

Schlegel, R. and Pardee, A. B. (1986). Caffeine-induced uncoupling of mitosis from the completion of DNA replication in mammalian cells. *Science* **232**, 1264-1266.

Schlegel, R. and Pardee, A. B. (1987). Periodic mitosis events induced in the absence of DNA replication. *Proc. Nat. Acad. Sci. USA.* **84**, 9025-9029.

Sheridan, W. F. (1982). *Maize for Biological Research*, pp. 1-434. University Press, University of North Dakota, Grand Forks, USA.

Staiger, C. J. and Cande, W. Z. (1990). Microtubule distribution in *dv*, a maize meiotic mutant defective in the prophase to metaphase transition. *Dev. Biol.* **138**, 231-242.

Staiger, C. J. and Cande, W. Z. (1991). Microfilament distribution in maize meiotic mutant correlates with microtubule organization. *Pl. Cell* **3**, 637-644.

Theurkauf, W. E. and Hawley, R. S. (1992). Meiotic spindle assembly in *Drosophila* females: behavior of nonexchange chromosomes and the effects of mutations in the nod kinesin-like protein. *J. Cell Biol.* **116**, 1167-80.

Tiwari, S. C. and Polito, V. S. (1990). The initiation and organization of microtubules in germinating pear (*Pyrus communis* L.) pollen. *Eur. J. Cell Biol.* **53**, 384-9.

West, D. P. (1985). The developmental basis of quantitative genetic variation revealed by the maize mutant, *Polymitotic*. Ph. D. thesis, University of Minnesota, USA.

West, D. P. and Phillips, R. L. (1985). Polymitotic:supernumerary repetitions of meiosis II. *Maize Genet. Coop. News Lett.* **59**, 106-107.

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