RESEARCH ARTICLE

Meiosis in a triploid hybrid of *Gossypium*: high frequency of secondary bipolar spindles at metaphase II

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Abstract

Studies on meiosis in pollen mother cells (PMCs) of a triploid interspecific hybrid ($3x$ = 39 chromosomes, AAD) between tetraploid *Gossypium hirsutum* ($4n = 2x$ = 52,AADD) and diploid *G. arboreum* ($2n = 2x$ = 26,AA) are reported. During meiotic metaphase I, 13 AA bivalents and 13 D univalents are expected in the hybrid. However, only 28% of the PMCs had this expected configuration. The rest of the PMCs had between 8 and 12 bivalents and between 12 and 17 univalents. Univalents lagged at anaphase I, and at metaphase II one or a group of univalents remained scattered in the cytoplasm and failed to assemble at a single metaphase plate. Primary bipolar spindles organized around the bivalents and multivalents. In addition to the primary spindle, several secondary and smaller bipolar spindles organized themselves around individual univalents and groups of univalents. Almost all (97%) of the PMCs showed secondary spindles. Each spindle functioned independently and despite their multiple numbers in a cell, meiosis I proceeded normally, with polyad formation. These observations strongly support the view that in plant meiocytes bilateral kinetochore symmetry is not required for establishing a bipolar spindle and that single unpaired chromosomes can initiate and stabilize the formation of a functional bipolar spindle.

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Introduction

Extensive chromosome reorganization and the formation of a single spindle during mitosis and meiosis are essential for proper chromosome segregation. Accurate segregation of chromosomes at meiosis I requires that homologous chromosomes pair with each other and form bivalents. A failure of homologues to pair and undergo recombination leads to inaccurate chromosome segregation at metaphase I, and the possible subsequent loss of unpaired chromosomes. During normal meiosis, it is the spindle that generates the forces to sort and segregate homologous chromosomes into daughter cells (reviewed in Franklin and Cande 1999). Despite much study, the exact mechanism of spindle formation continues to be debated. There are two models to explain spindle assembly: the 'search and capture' model and the 'self assembly' model (Chan and Cande 1998). In the search and capture model, the bipolarity of the spindle pole, and the orientation of spindle microtubules, are generated by newly separated centrosomes even before nuclear membrane breakdown occurs. The minus ends of microtubules are at the centrosomes and their plus ends 'search and capture' kinetochores, thus stabilizing the plus ends. This mode of spindle assembly is observed in several animal species (Vernos and Karsenti 1995).

Plant cells totally lack conspicuous centrosome assemblies, and it has been observed that after nuclear envelope breakdown, an extensive cytoplasmic array of microtubules nucleate condensed chromatin (Mazia 1984; Chan and Cande 1998; Franklin and Cande 1999). The microtubules later rapidly self-organize into a bipolar array, and the spindles grow with time to reach the cell membrane. Chan and Cande (1998) also observed that bilateral kinetochore symmetry of chromosomes at metaphase I in maize pollen mother cells (PMCs) is not required for the establishment of a bipolar

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spindle, because bipolar spindles have been observed even in desynaptic mutants with univalents. Several mutations affecting chromosome segregation in maize and other species are reported, but there are very few that directly affect spindle formation (Golubovskaya 1989). It would be useful to understand this biological process more deeply to be able to manipulate chromosome segregation and cytokinesis. Meiosis in aneuploid plants offers ample opportunity for the study of spindle organization and cytokinesis in the absence of normal chromosome pairing. We report here studies on chromosome pairing, spindle formation and cytokinesis in PMCs of a triploid interspecific hybrid of *Gossypium* spp.

Materials and methods

Raising plants

Plants of Asiatic diploid cotton *G. arboreum* cv. Shyamli ($2n = 2x = 26$,AA) and American upland tetraploid cotton *G. hirsutum* cv. L604 ($4n = 2x = 52$,AADD) were sown in I.A.R.I. (New Delhi, India) fields during 2001–2002 and 2002–2003 in a randomized block design with three replications in 4.2-m rows with a row spacing of 75 cm. Plant spacing within the row was approximately 30 cm. Standard agronomic and plant protection practices were followed.

Production of triploid hybrids

G. hirsutum cv. L604 was used as the female parent and *G. arboreum* cv. Shyamli as the male parent. Flower buds on the maternal plant were hand-emasculated at appropriate stages of development during late evenings, and pollinated the next morning. Naphthalene acetic acid (100 mg/1) and gibberellic acid (50 mg/l) were sprayed at the base of the pedicle for 5 or 6 days after pollination to reduce boll shedding. Hybrids were raised by serial ovule and embryo culture *in vitro*. The hybrid seedlings derived from embryos germinated *in vitro* were raised under controlled conditions in The National Phytotron Facility of I.A.R.I. in 2002.

Meiosis

Flower buds were collected twice a day (from one hybrid plant), fixed in 95% ethanol : glacial acetic acid (3:1) for one week, washed with distilled water, and preserved in 70% ethanol until use. Chromosomes were studied in temporary iron–aceto-orcein squashes of PMCs. Selected chromosome spreads were photographed with a Nikon E 600 photomicroscope.

Observations on chromosome pairing at various stages of meiosis were recorded. Pollen fertility was estimated using aceto-orcein : 50% glycerin (1:1). Completely round and well-stained pollen grains were considered fertile, and those that were unstained and distorted in shape were taken as infertile.

Results

The AADD tetraploid parent, *G. hirsutum*, showed 26 bivalents (figure 1,A&B) and normal segregation of bivalents at anaphase I (figure 1,C) and of chromatids at anaphase II (figure 1,D). Pollen tetrads were normal (figure 1,E&F) and fertility was 95%. Similarly, the diploid parent showed 13 bivalents (figure 1,G) at metaphase I and exhibited normal pollen grain formation.

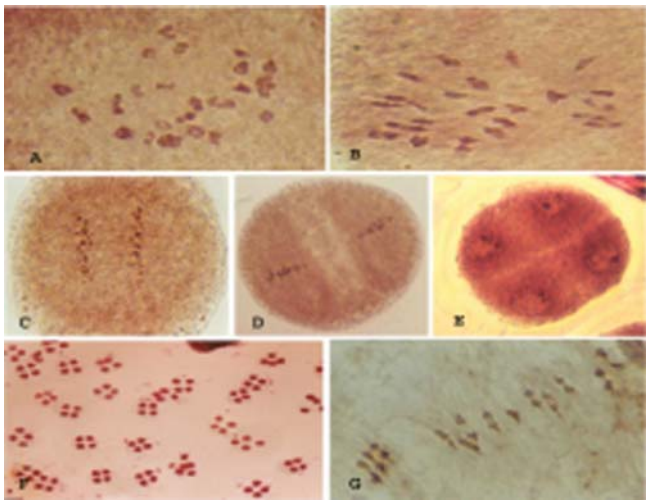

Figure 1. Meiosis in PMCs of *Gossypium*. (A–F), *G. hirsutum* cv. L604 ($4x$, $2n = 52$,AADD). (A, B) Metaphase I (26 bivalents); (C) anaphase I, single bipolar spindle; (D) metaphase II, two bipolar spindles; (E, F) pollen tetrads. (G) *G. arboreum* cv. Shyamli ($2x$, $2n = 26$,AA); metaphase I with 13 A bivalents.

The PMCs of the hybrid showed 39 chromosomes (26 A, 13 D), thus confirming its hybrid as well as triploid nature. According to theoretical expectations, there ought to be 13 AA bivalents and 13 D univalents at metaphase I in this hybrid . However, the theoretically expected $13_{II} + 13_{I}$ configuration was observed only in 25.8% of PMCs. Univalent, bivalent, trivalent, quadrivalent and hexavalent chromosome associations were recorded at various frequencies among the 309 PMCs (table 1; figure 2,A–D). Univalents ranged from 12 to 17 in number per PMC, and about 78.9% of PMCs had 13 univalents, 16.8% had more than 13 univalents, and 4.3% had less than 13 univalents. The number of bivalents ranged from 8 to 13; 74.2% of PMCs had 8–12 bivalents and 27% had 10 bivalents. Trivalents and quadrivalents ranged from zero to one per PMC, and the frequencies of PMCs with trivalents and quadrivalents were 6.2% and 34.4% respectively (table 1). Quadrivalents and hexavalents usually showed adjacent and alternative associations. The $13_{I} + 10_{II} + 1_{IV}$ arrangement at metaphase I was observed in 23% of PMCs. Configurations with both trivalents and quadrivalents, or trivalents and hexavalents, were observed in 3.3% of PMCs. Overall, the 17_{I} and 11_{II} configuration was the least prevalent. At anaphase I, laggard chromosomes and

Table 1. Chromosome associations at metaphase I in 309 PMCs of the triploid F_1 derived from *G. hirsutum* and *G. arboreum.*

Type and no. of associations per PMC					PMCs	
					Number	%
I	II	III	IV	VI		
13	13				54	25.8
13	8		1	1	27	12.9
13	10			1	48	23.0
13	11		1		36	17.2
12	12	1			6	2.9
15	10		1		6	2.9
15	12				24	11.5
17	11				1	0.5
12	10	1	1		3	1.4
14	8	1		1	4	1.9

I, Univalent; II, bivalent; III, trivalent; IV, quadrivalent; VI, hexavalent.

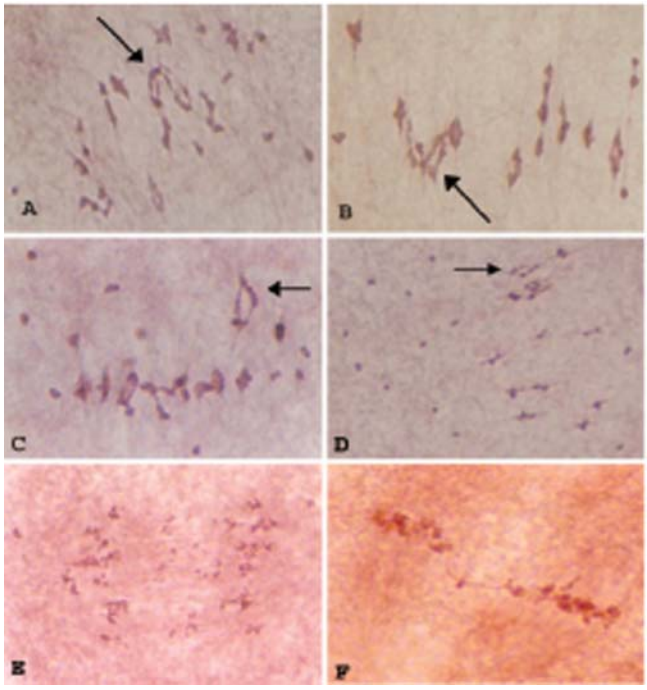

Figure 2. Chromosomes at metaphase and anaphase in PMCs of the F_1 hybrid *G. hirsutum* × *G. arboreum* ($3x, 2n = 39$,AAD). (A–D) Metaphase I; (E) anaphase I; (F) metaphase II. (A) 9 II, 2 III, 1 VI, 6 I (3 I were out of the field), ×1000, arrow shows VI; (B) 8 II, 1 IV, 1 III, 2 I (15 I were out of the field) ×1200, arrow shows IV; (C) 5 II, 1 III, 2 IV, 11 I (8 I were out of the field), ×1000, arrow shows IV; (D) 12 II, 1 IV, 11 I, ×700, arrow shows IV; (E) laggards at anaphase I; (F) bridge at metaphase II.

bridges were frequently observed (table 2; figure 2,E&F). The number of laggard chromosomes per PMC ranged from zero to 10.

Two bipolar spindles are expected during normal metaphase II. In this interspecific hybrid, the bivalent and multivalent chromosomes that had sorted into opposite poles during anaphase I and telophase I organized into normal bipolar spindles at metaphase II. The univalent chromosomes that lagged at anaphase I formed random chromosome clusters consisting of variable numbers, usually between 1 and 5, of univalents (figure 3,B). At metaphase II, supernumerary bipolar spindles organized around these univalent chromosome clusters (figure 3,C–F). Only 3.2% of PMCs showed two bipolar spindles. The remaining 97% of PMCs showed 1–4 supernumerary spindles per cell, apart from the two primary bipolar spindles (table 3; figure 3,C–F). Overall, 45.2%, 32.3%, 13% and 6.5% of PMCs showed one, two, three and four supernumerary spindles respectively . The supernumerary spindles generally had fewer chromosomes than the primary spindle, and their chromosome numbers ranged from as low as one chromosome per spindle to as many as 13 per spindle (table 4). The supernumerary spindle(s), irrespective of the number of chromosomes, appeared normal and proceeded to function normally and independently through anaphase II. They occupied mutually exclusive cytoplasmic spaces within the PMC. While the two primary spindles followed identical polarity, the secondary spindles were almost always differently oriented with respect to the primary spindles. The presence of multiple spindles, however, did not affect the function of the primary or secondary spindles. Meiosis proceeded through to telophase II and pollen grain formation, regardless of the chromosome numbers per pole or their orientation. Dyad, triad, pentad and hexad pollen grain clusters were observed as a consequence of the extra daughter cells formed from the supernumerary chromosome clusters (figure 3,G&H). Triads were observed at a frequency of 18.9%, and pentads and hexads at a frequency of about 49% (table 5). The pollen grains varied greatly in size and were completely sterile, compared to the *G. hirsutum* and *G. arboreum* parents that showed 95–98% pollen fertility.

Table 2. Chromosome associations at anaphase I in 32 PMCs of the triploid F_1 hybrid between *G. hirsutum* and *G. arboreum*.

Nos. of chromosomes at each pole	No. of laggards	No. (%) of PMCs
13 + 17	9	6 (18.8)
13 + 23	3	5 (15.6)
13 + 19	7	5 (15.6)
13 + 20	6	4 (12.5)
14 + 15	10	4 (12.5)
16 + 20	3	3 (9.4)
15 + 18	6	3 (9.4)
23 + 16	0	2 (6.3)

Discussion

Spindles are bipolar structures, and their bipolar symmetry is essential for chromosome segregration. Mazia (1984) suggested that a single, flexible centrosome spreads throughout

Table 3. Frequency of bipolar spindles at anaphase II in PMCs (n = 31) of the triploid F_1 hybrid between *G. hirsutum* and *G. arboreum*.

	No. of bipolar spindles per PMC				
	2	3	4	5	6
No. of PMCs	1	14	10	4	2
%	3.2	45.2	32.3	12.9	6.5

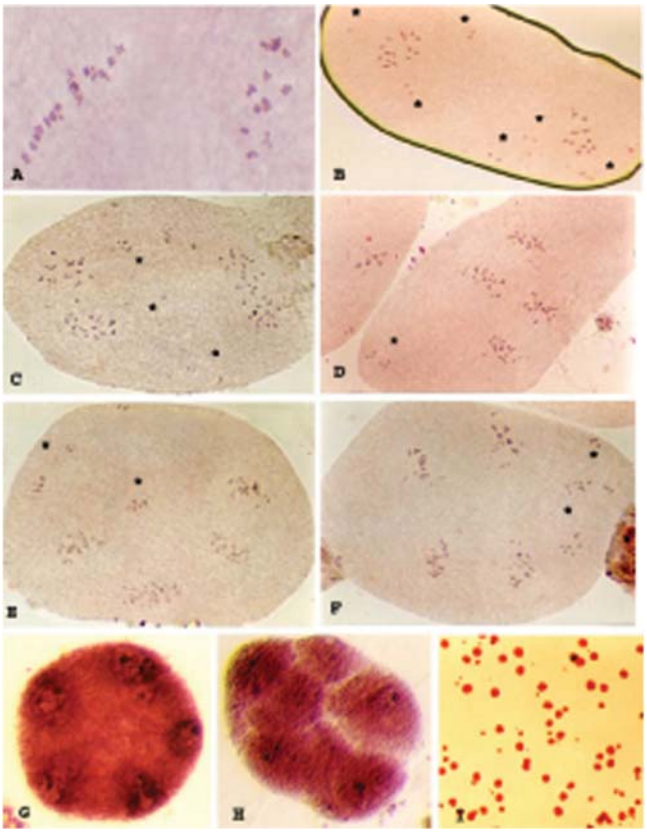

Figure 3. (A–E) Metaphase II and anaphase II in triploid F_1 hybrid (*G. hirsutum* × *G. arboreum*). (A) Metaphase II with two primary chromosome clusters (×1200); (B) metaphase II with two primary clusters and six secondary clusters (×800) (* shows secondary clusters); (C–F) anaphase II with 3, 1, 2 and 3 supernumerary spindles in the same PMC (×800) (* shows supernumerary spindles); (G,H) clusters of 5 and 7 pollen grains; (I) sterile pollen grains of varying sizes.

the plant cell and nucleates microtubules at specific times and specific sites. An alternative view is that plants do not have a centrosome-like structure and that the meiotic plant spindle poles are initiated from the chromosomes by self-assembly. The self-assembly process is thought to begin at the kinetochore, when condensed chromatin attaches to microtubule fibres (Baskin and Cande 1990; Smirnova and Bajer 1994, 1998; Waters and Salmon 1997). The spindles then assemble around the mass of chromosomes, and initially appear as poorly organized, often multipolar, structures (Yu *et al*. 1999). Bipolar meiotic spindles merge at mid-prometaphase in both meiosis I and meiosis II (Staiger and Cande 1990). The progressive self-organization of the focussed bipolar spindle probably occurs through the combined effects of microtubule bundling and specific motor activities (Waters and Salmon 1997). In *Lilium longiflorum*, for example, bipolarity of spindles is never observed before the breakdown of the nuclear envelope. Prior to prometaphase I, several foci appear to be centres of microtubule organization. After breakdown of the nuclear envelope, the microtubules around the nucleus invade the regions between the chromosomes. Multiple spindle poles are then visible at early prometaphase I. Bipolarity of the spindle seems to become established gradually as prometaphase I progresses. During mid-prometaphase I, the multiple spindle poles become concentrated into two poles and the spindle axis becomes fixed. The two spindle poles gradually become focussed, and the typical meiotic spindle is complete at metaphase I (Suzuki *et al*. 1997). In contrast, maize (Staiger and Cande 1990), like some lilies (Sheldon and Hawes 1988) and the orchid *Cypripedium californicum* (Brown and Lemmon 1998), differs in that the spindle is approximately bipolar from inception and does not pass through a multipolar stage. At the origination of the meiosis I spindle in wild–type maize, perinuclear microtubules increase around the nucleus, which becomes elongated along the axis of the forming spindle. At nuclear envelope breakdown, a bipolar spindle engages the chromosomes (Brown and Lemmon 1998). In *C. californicum*, the microtubules of the forming spindle are divided into two arrays that extend from the two nuclear hemispheres (Brown and Lemmon 1998), whereas in the meiotic spindle of *Lilium* spp., two opposing systems of microtubules emanate from definite sites at the surface of the nuclear envelope in the zygotene stage (Sheldon and Hawes 1988).

In the present study of meiosis in PMCs of a triploid

Table 4. Chromosomes in supernumerary spindles at anaphase II in PMCs (n = 54) of the triploid F_1 hybrid between *G. hirsutum* and *G. arboreum*.

	No. of chromosomes per supernumerary spindle												
	1	2	3	4	5	6	7	8	9	10	11	12	13
No. of PMCs	2	3	7	6	3	8	6	4	6	5	1	1	2
%	3.7	5.6	13.0	11.1	5.6	14.8	11.1	7.4	11.1	9.3	1.9	1.9	3.7

Table 5. Pollen cluster combinations among PMCs (n = 11) in the triploid F_1 hybrid between *G. hirsutum* and *G. arboreum*.

Pollen cluster combinations	PMCs	
	Number	%
Triads	21	18.9
Tetrads	35	31.5
Pentads and hexads	55	49.5

Gossypium (AAD) hybrid, a large number of unpaired univalent chromosomes that did not segregate at metaphase I remained in single or random clusters in the meiocyte cytoplasm. At anaphase I, only synapsed chromosomes segregated to the poles, suggesting that spindle fibres did not attach to univalents at metaphase I. In contrast, the univalent chromosome(s) promoted the nucleation of supernumerary spindle fibres to organize multiple minispindles at metaphase II in addition to the two primary spindles that organized around the normally segregated bivalent and multivalent chromosomes. Even a single univalent was sufficient to organize a minispindle. The abnormality described here affected up to 97% of the PMCs. The primary and secondary spindles were rarely of identical polarity and occupied mutually complementary and nonoverlapping spaces within the PMC. Despite the large numbers of spindles per cell, and the high proportion of PMCs with secondary spindle formation, the chromatin cycle appeared synchronous, and meiosis II was completed. This clearly suggests that the secondary spindles not only did not interfere with the function of the primary spindles, but also responded to cell cycle cues in synchrony with the primary spindles. The occurrence of multiple spindles very strongly suggests that *Gossypium* does not possess centrosome-like structures that organize a single bipolar spindle. Microtubule networks (remaining from meiosis I in this case) appear to be spread randomly in the cytoplasm and are capable of forming normal multiple bipolar spindles of diverse polarities when presented with chromatin. Polyads, and microspores with micronuclei and reduced pollen fertility, were the most prominent manifestations of this irregularity and led to complete pollen sterility. This situation also offers useful opportunities to study the control over postmeiotic cytokinesis and cell wall formation.

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