Heterochronic Expression of Sexual Reproductive Programs During Apomictic Development in Tripsacum

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ABSTRACT

Some angiosperms reproduce by apomixis, a natural way of cloning through seeds. Apomictic plants bypass both meiosis and egg cell fertilization, producing progeny that are genetic replicas of the mother plant. In this report, we analyze reproductive development in *Tripsacum dactyloides*, an apomictic relative of maize, and in experimental apomictic hybrids between maize and Tripsacum. We show that apomictic reproduction is characterized by an alteration of developmental timing of both sporogenesis and early embryo development. The absence of female meiosis in apomictic Tripsacum results from an early termination of female meiosis. Similarily, parthenogenetic development of a maternal embryo in apomicts results from precocious induction of early embryogenesis events. We also show that male meiosis in apomicts is characterized by comparable asynchronous expression of developmental stages. Apomixis thus results in an array of possible phenotypes, including wild-type sexual development. Overall, our observations suggest that apomixis in Tripsacum is a heterochronic phenotype; *i.e.*, it relies on a deregulation of the timing of reproductive events, rather than on the alteration of a specific component of the reproductive pathway.

SEXUAL reproduction in angiosperms occurs within a highly differentiated multicellular structure, the ovule. The formation of the female gametes within the ovules entails two consecutive steps: megasporogenesis (spore formation) and megagametogenesis (gamete formation). Megasporogenesis initiates with the formation of the megaspore mother cell (MMC), which undergoes meiosis. Meiosis results in the production of four megaspores, containing half the number of chromosomes of the sporophyte. In most angiosperms, three of the four spores degenerate, leaving a single functional megaspore. During megagametogenesis, the megaspore undergoes mitotic divisions, typically three rounds, producing a multicellular gametophyte (the embryo sac) containing the gamete (the egg cell). In the most common type of gametophyte development in plants (the Polygonum type), the mature gametophyte contains one single egg cell, a central cell, two synergids at the micropylar pole, and a variable number of antipodal cells at the chalazal pole.

In male reproductive organs (the anthers), all four products of meiosis survive. They divide and differentiate to produce the male gametophyte (the pollen grain), which contains two reproductive sperm cells, both of which are involved in the double fertilization of the female gametophyte. The fertilization of the egg cell by one of two sperm cells leads to the formation of the embryo, while the fertilization of the central cell by the second male sperm cell gives rise to the endosperm. The central cell contains two nuclei. The endosperm and the embryo therefore have different ploidy levels: $3x$ for the endosperm and $2x$ for the embryo.

Gametophytic apomixis (referred to hereafter as apomixis) is a process of asexual reproduction through seeds (Nogler 1984). Apomictic plants bypass both meiotic reduction (a process called apomeiosis) and egg-cell fertilization, thus producing offspring that are exact genetic replicas of the mother plant. In the diplosporous type of apomixis, a normal MMC differentiates, but fails to complete meiosis; the process either aborts in metaphase or anaphase I (defining the Taraxacum type of diplospory) or is omitted altogether (defining the Antenaria type of diplospory). The resulting unreduced megaspore then develops through mitoses into an embryo sac that contains unreduced nuclei. The embryo develops from the egg cell without fertilization, *i.e.*, by parthenogenesis. The endosperm can be derived either after fertilization of the polar nuclei, and is therefore sexual (pseudogamous apomixis), or without fertilization of the central cell, *i.e.*, parthenogenetically (autonomous apomixis). Male meiosis in apomicts usually results in functional, reduced male gametophytes. Furthermore, apomixis is typically a facultative phenomenon in which plants produce both reduced and unreduced megagametophytes in variable proportions. Thus, apomictic plants conserve an unaltered potential for male and female meiosis.

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Apomixis has been intensively studied at the structural level (Nogler 1984; Asker and Jerling 1992; Savidan 2000). Despite decades of research, though, our understanding of the cellular and molecular mechanisms of apomixis is still in its infancy. The limited data derived from genetic analyses suggest that apomicts arose from sexual ancestors through a limited number of mutations in the female reproductive pathway (reviewed in Nogler 1984; Koltunow 1993; Grimanelli *et al.* 2001; Grossniklaus *et al.* 2001). The underlying genes are unknown as yet, but possible modes of action have been proposed. One hypothesis is that apomeiosis results from an early induction of the transcriptional cascade responsible for embryo sac development; expression in the MMC prior to or early in meiosis would lead to unreduced embryo sac formation. Alternatively, it has been proposed that apomeiosis might result from a shift in cell fate within the ovule: the MMC would turn into a "spore identity" and thus onto a mitotic embryo sac development pathway prior to entering meiosis. Parthenogenesis, on the other end, is often viewed as a pleiotropic consequence of apomeiosis. The strongest argument comes from genetic experiments. They show that both apomeiosis and parthenogenesis are usually fully linked in segregating populations, suggesting that a common set of mutations is involved (Nogler 1984; Grossniklaus *et al.* 2001).

Here, we tested these hypotheses by analyzing diplosporous apomictic development in *Tripsacum dactyloides* L., a wild relative of maize (*Zea mays* L.), and in hybrids between apomictic Tripsacum and sexual maize plants. Our analyses suggest a more complex picture: apomixis in Tripsacum and maize-Tripsacum hybrids is characterized by an alteration of the developmental timing of sporogenesis and early embryo development, rather than by gametogenesis. We further demonstrate that the precocious initiation of embryo development can occur regardless of meiotic reduction and thus is not a direct consequence of the absence of meiosis. We suggest that apomixis in Tripsacum is a typical heterochronic phenotype: it results from the temporal alteration of the orderly progression of the developmental subroutines that constitute the sexual pathway, but without disruption of the subroutines themselves.

MATERIALS AND METHODS

Plant materials: We used two maize inbred lines, CML135 and CML139, and hybrids (referred to herein as the H1 hybrid) between those two lines as controls for wild-type sexual development in maize. A diploid, sexual *T. dactyloides* plant, accession BT-FCM, was used as a reference for sexual development in Tripsacum. Four apomictic accessions (nos. 65-1234, 11-36, 61-664, and 112-1327) of tetraploid *T. dactyloides* were obtained from the CIMMYT germplasm bank (http://www.cimmyt.org). They have been previously characterized for their mode of reproduction (Leblanc *et al.* 1995) and conserved as perennial materials in field conditions in Mexico. Apomictic accessions of Tripsacum are all polyploids, pseudogamous, and reproduce via the Antenaria type of diplospory.

Various generations of maize-Tripsacum hybrids and hybrid derivatives were obtained with Tripsacum accession 65-1234. Pedigrees and a precise description of their reproductive behavior can be found elsewhere (Leblanc *et al.* 1996). This article uses one such hybrid genotype, referred to as 38C. It contains a diploid set of maize chromosomes ($2x = 20$) and one haploid set of Tripsacum chromosomes ($x = 18$). It reproduces by diplosporous apomixis. The 38C material originally consisted of a unique plant. Clones of the original plant were multiplied through apomixis. Expression of diplospory was estimated from seeds using a flow cytometry procedure as described (Leblanc *et al.* 1996).

Cytological characterization of male and female meiocytes: Female and male meiocytes were collected at various stages before tassel and ear emergence, fixed in a mix of ethanol:chloroform:acetic acid (6:3:1) for 24 hr, and stored in 75% ethanol. For male meiocytes, we fixed individual anthers after dissection from the florets. Chromatin was stained with 20 μl of 1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) in 1× PBS buffer.

For female meiocytes, entire developing ears were fixed. We sampled for each material a total of 12 ears representing different stages of development from MMC differentiation (ears 1.5 cm long) to mature embryo sacs. The ovules were dissected, positioned in a 96-well plate to reflect their position on the ear, and analyzed individually. A minimum of 12 ovules were analyzed per sample. Stages of ovule development were observed in half of the ovules through whole-mount cleared ovules, while chromosome configuration was analyzed using DAPI staining for the other half of the ovules. For DAPI staining, ovules were extruded from the ovaries, digested for 15–30 min using a mix of 5% cellulase and 1% pectolyase in 0.01 m citrate buffer, washed twice in water, and placed on a slide. They were gently squashed to separate the cells and stained with DAPI. Preparations were observed directly with epifluorescence on a Leitz Aristoplan microscope. Ovule clearing was performed using a 2.1:1 solution of benzyl-benzoate:dibutylphtalate clearing solution as previously described (Leblanc *et al.* 1995) and observed with differential interference contrast optics.

Ploidy levels of mature pollen grains were estimated using flow cytometry. Pollen grains were extruded from freshly harvested anthers and directly analyzed using the same protocol used for leaf tissues. For each sample, five replicates consisting of a bulk of 10 different anthers were analyzed using a PARTEC CAII flow cytometer. A diploid maize line (CML216) was used as a standard in all measurements. Five thousand nuclei were counted for each entry.

Immunofluorescence: Anthers of the appropriate stages were placed into 2 ml of fixative solution [8% (v/v) paraformaldehyde] and PHEMS buffer (60 mm Pipes, 25 mm HEPES, 10 mm EGTA, 2 mm $MgCl_2$, and 0.32 m sorbitol, pH 7.2) for 30 min, rinsed, and stored in PHEMS buffer. Meiocytes were extruded from the anthers in a drop of 1× PBS and embedded in polyacrylamide following Bass *et al.* (1997). Such fixation and embedding procedures have been previously shown to maintain both native chromatin structure and the three-dimensional architecture of the nucleus. Acrylamide pads were incubated for 20 min in 1% β-glucanase to partially digest the callose walls surrounding the male meiocytes. The cells were then permeabilized for 2 hr in 1× PBS, 1% Triton X-100. Samples were incubated overnight in a humid chamber with 50 μl of either a 1:500 dilution of an antitubulin antibody (Sigma, St. Louis) or a 1:200 dilution of an antibody that recognizes a ser10 phosphoepitope on histone H3 (Upstate

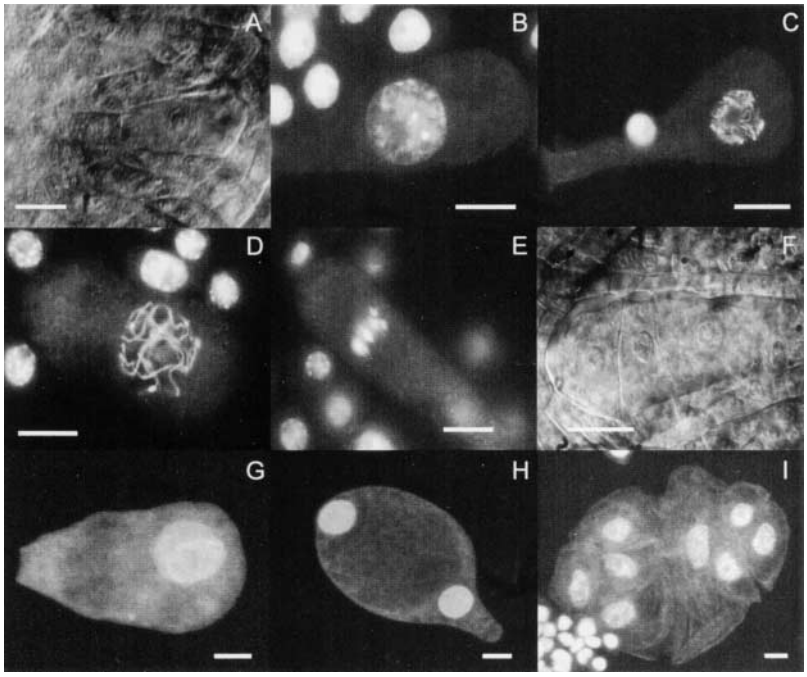


Figure 1.—Megasporogenesis and megagametogenesis in sexual maize and Tripsacum plants. (A) Whole-mount ovule clearing of a differentiated MMC at the onset of meiosis in Tripsacum. (B) DAPI staining of an isolated MMC at a similar stage in maize. (C–I) Isolated female meiocytes and embryo sacs from Tripsacum ovules; isolated meiocyte at leptotene (C), pachytene (D), and metaphase (E). (F) Whole-mount ovule clearing at the tetrad stage. (G–I) Development of the embryo sac at the uninuclear (G), binucleate (H), and eight-nucleate stages (I). Bar, 25 um.

Biotechnology, Lake Placid, NY). Slides were then washed five times, 1 hr each, in 1× PBS, 0.1% Triton X-100. A FITC-conjugated donkey anti-rabbit (phospho H3) or anti-mouse (tubulin) antibody (Sigma) at a dilution of 1:200 in PBS buffer was incubated overnight, and the same washing protocol was followed the next day. Samples were stained with 50 μl of 1 mg/ml propidium iodide or 1 mg/ml DAPI for 30 min, washed for 30 min in 1× PBS, mounted in an antifading solution (Vectashield), and sealed. Three-dimensional fluorescence images were collected on a cooled CCD camera (Cooke SensiCam QE). Three-dimensional images of the meiocytes were captured and processed with the SlideBook 3D System (Intelligent Imaging Innovations). Image deconvolution was performed with the Nearest Neighbors algorithm, and two-dimensional images were generated using maximum intensity projections of selected optical sections.

Embryo development: Maize, Tripsacum, and maize-Tripsacum hybrid materials were used to visualize timing of embryo development with and without fertilization in apomictic and sexual materials. For each, female inflorescences were pollinated under controlled conditions using H1 as a pollen donor. Immature ears were collected at 0, 8, 12, and 24 hr and at 2, 4, and 5 days after pollination (DAP). Nonpollinated materials were also collected by covering flowers to avoid pollination and collecting at an equivalent estimated time based on the timing of silk emergence. The samples were embedded in paraffin, sectioned, and stained using standard procedures. Images were collected with a CCD camera. Image manipulation and editing was performed using the GraphicConverter software (lemkesoft.com). DNA fingerprinting of seedlings was performed using a nonradioactive amplified fragment length polymorphism (AFLP) protocol (Hoisington *et al.* 1994), which allows for the rapid generation of a large number of polymorphic alleles.

RESULTS

Megasporogenesis and megagametogenesis in maize and sexual Tripsacum: Megasporogenesis and megagametogenesis in maize, which follows the Polygonum type of gametophyte development, have been described in various publications (Russell 1978; Golubovskaya *et al.* 1992; Huang and Sheridan 1996). Our materials did not differ notedly from previous descriptions. Megasporogenesis and megagametogenesis also appeared similar in sexual maize and sexual Tripsacum, two closely related species (Figure 1).

In addition, we looked at the synchronization of MMC and the ovule development in individual ovaries and between ovaries on the same ear. In maize, timing of ovule development and meiosis are synchronized. On the ears, they followed a regular gradient, with the most advanced ovules being located at the bottom of the ear and the youngest cells at the top. At a given height on the ear, "rings" of ovaries presented both synchronized ovules and synchronized meiocytes. Hence, development stages of the meiocytes can be predicted from the stages of ovule development, and reciprocally. In Tripsacum, ears were smaller and contained a limited number of female flowers, 15–25 in our accessions. All ovules and meiocytes on a given ear were found at very similar stages of development throughout female reproductive development.

Megasporogenesis and megagametogenesis in apomictic Tripsacum: Diplospory was analyzed in a single entry of *T. dactyloides*, accession no. 65-1234. Ovule growth followed a path similar to that observed in maize and sexual Tripsacum. Synchronized ovules were observed on the ears all the way from MMC differentiation to mature ovaries. Contrary to the ovules, however, meiocytes were not synchronized. Chromatin staining of meiocytes after MMC differentiation identified two classes of cells. The first class (half of the cells, Table 1) contained cells in interphase. The second class con-

TABLE 1

Occurrence of meiosis in apomictic Tripsacum (accession 65-1234) and maize-Tripsacum hybrids (genotype 38C)

	Interphase nucleus	Meiosis I: leptotene-zygotene	Meiosis I: pachytene	Meiosis I: >pachytene	Meiosis II	Mitotic prophase	Embryo sacs: 1–8 nuclei	Total
Tr. 651234, stage 1	36	0	0	0	0	0	30	66
Frequency	0.55	0.00	0.00	0.00	0.00	0.00	0.45	1.00
38C, stage 1	55	17	6	0	0	0	0	78
Frequency	0.71	0.22	0.08	0.00	0.00	0.00	0.00	1.00
38C, stage 2	0	0	0	0	10	12	61	83
Frequency	0.00	0.00	0.00	0.00	0.12	0.14	0.73	1.00
38C, stage 3	0	0	0	0	3	2	22	27
Frequency	0.00	0.00	0.00	0.00	0.11	0.07	0.81	1.00

Stage 1 corresponds to ovules at early meiotic stages in sexual maize and Tripsacum plants. Stage 2 corresponds to ovules at the end of prophase I in sexual materials. Stage 3 corresponds to ovules in meiosis II in sexual plants.

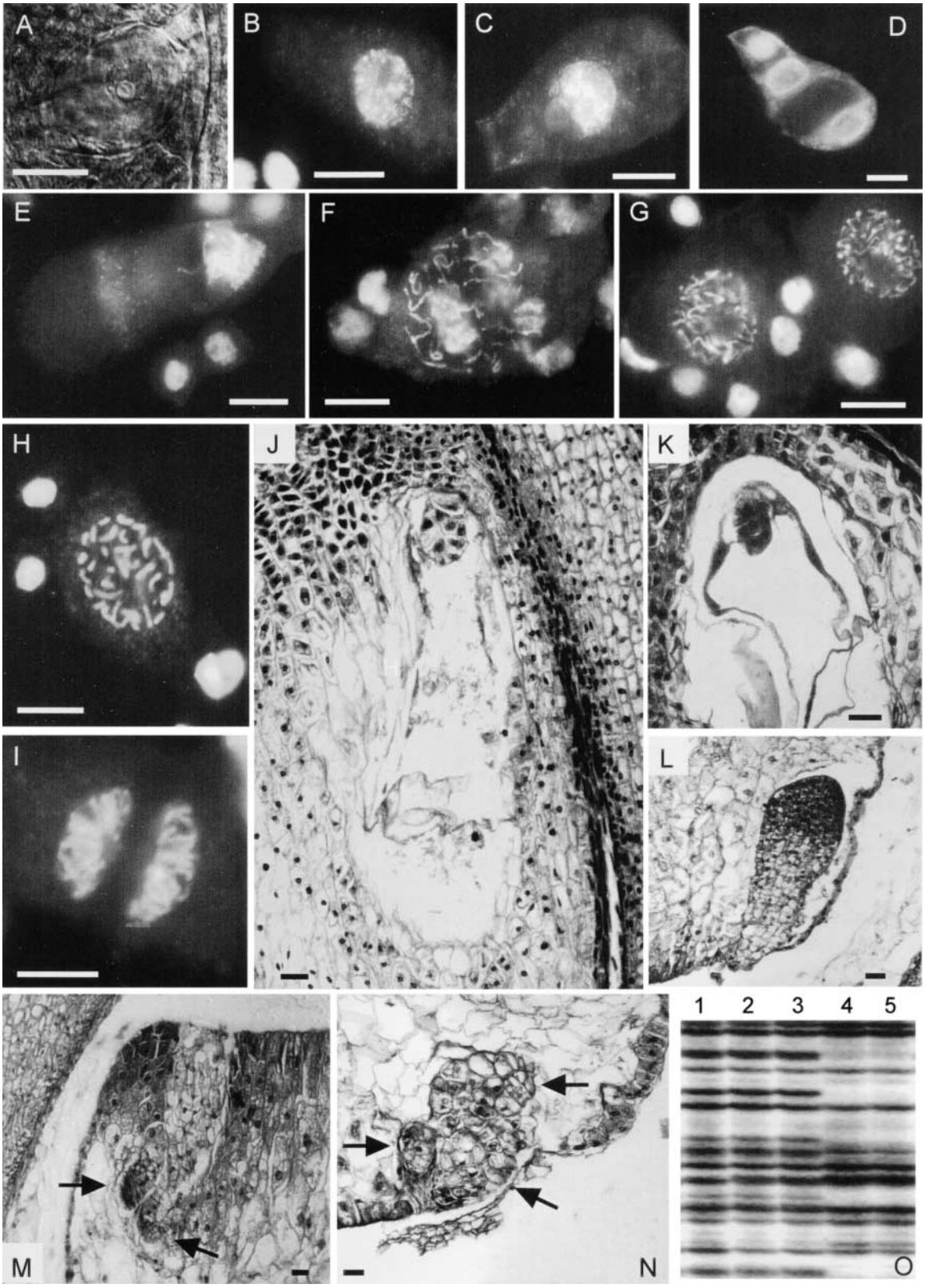
tained developing embryo sacs. Those could be differentiated by their size, shape, and the presence of a large central vacuole (Figure 2). Ears analyzed at later stages of ovule development were all found to contain synchronized ovules, but embryo sacs of various maturity. No aborted ovules were observed at that stage, indicating that asynchronous development does not result in developmental arrest. Although the observation of ovule development stages indicates that our sampling covered extensively the period following MMC differentiation, meiotic chromosomes were never observed.

Megasporogenesis in maize-Tripsacum hybrid derivatives: We first estimated the frequency of embryos derived through diplospory in 38C clones. All visible embryos (252 out of a total of 267 flowers or 94%) were extracted from three mature ears and analyzed for mode of reproduction using flow cytometry. Overall, 98% of the embryos originated from unreduced gametes (fertilized or not). The remaining 2% originated from sexual reproduction.

Ovule clearing showed that, as in maize, ovule development was synchronized on the ear. Surprisingly, ~30% of the meiocytes extracted after MMC differentiation displayed meiotic chromosome configurations with DAPI staining (Table 1; Figure 2). Early meiotic stages, leptotene or zygotene, were observed. No sample later than pachytene was recorded. The remaining meiocytes at similar stages of ovule development were differentiated, uninucleate embryo sacs similar to those found in Tripsacum. Ears sampled later during development, corresponding to ovules in late prophase I or meiosis II in sexual materials, also showed asynchronous meiocyte development (Table 1): while a majority of the cells were embryo sacs at various stages of development, 26% displayed either mitotic-like prophase (showing neither the chromosome pairing nor the typical distribution of chromosomes and organelles that are characteristic of female meiosis I) or dyads of individualized cells (Figure 2, F and G, respectively). Chromosome counts indicate that both cells in the dyads contained 38 chromosomes, similar to the mother plant (Figure 2). Such genomic constitution, consisting of two maize genomes ($2x = 20$) and one haploid Tripsacum genome ($x = 18$) is highly unstable through meiosis, as shown elsewhere (Grimanelli *et al.* 1998). The presence of two identical cells with 38 chromosomes shows that the dyads resulted

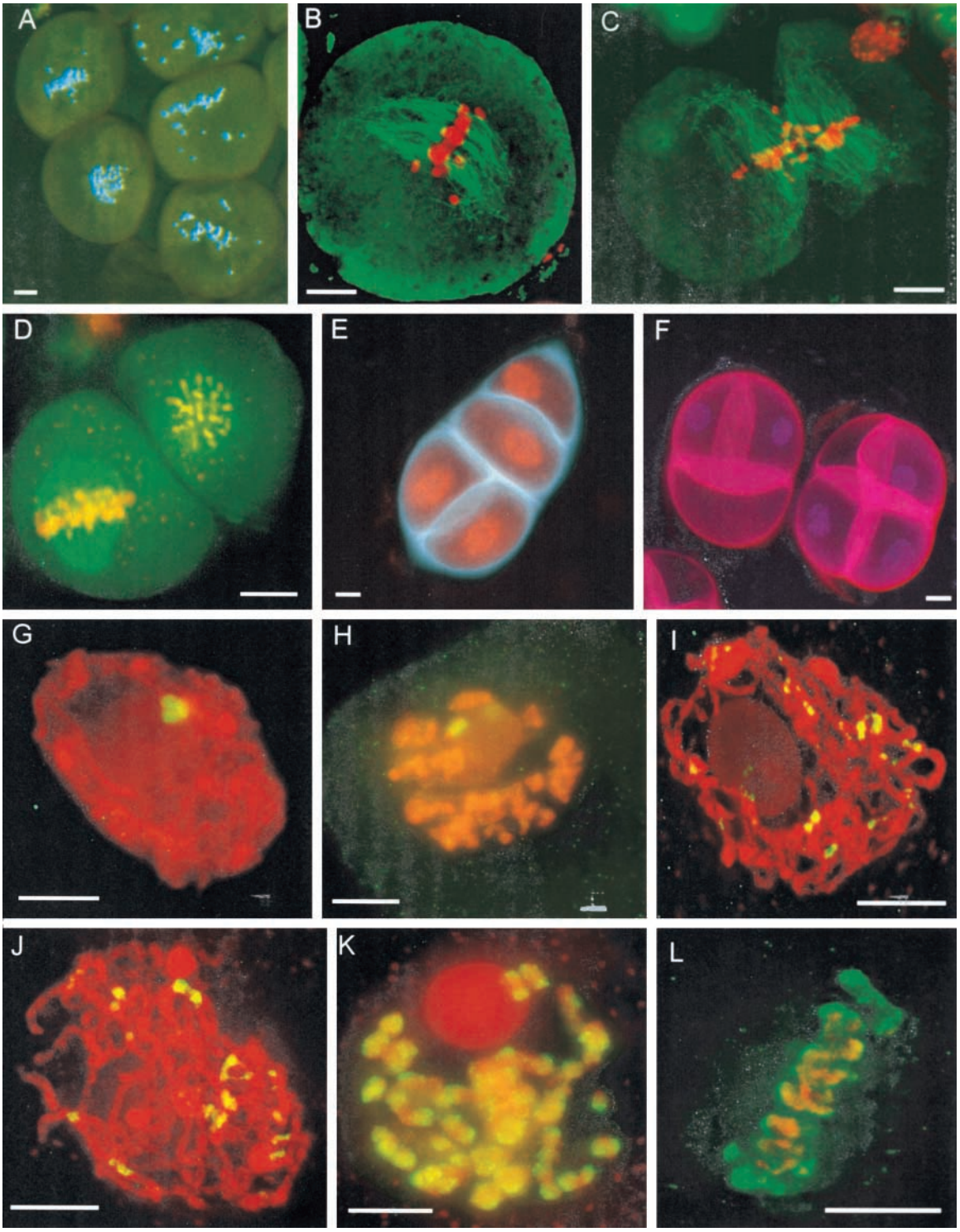
Figure 2.—Spore, gamete, and embryo development in apomictic Tripsacum and maize-Tripsacum hybrids. (A) MMC in Tripsacum 65-1234. Note the difference in shape from Figure 1A, due to the absence of rigid callose walls in the apomicts (Leblanc *et al.* 1995). (B–E) Isolated meiocytes from ovules dissected at similar stages of development in maize-Tripsacum hybrid 38C, corresponding to early meiosis in sexual plants. (B) Isolated MMC. (C) Uninucleate embryo sac. (D) Binucleate embryo sac. (E) Meiotic configuration. (F–I) Isolated meiocytes from ovules dissected at similar stages of development in 38C, corresponding to metaphase I in sexual plants. (F) End of mitotic prophase. (G) Dyad of mitotic "sister cells," both with an identical number of 38 chromosomes, similar to that of the mother plant. (H) Mitotic prophase, with 38 unpaired chromosomes, similar to that of the mother plant. (I) Mitosis. (J) Section of an embryo sac prior to fertilization in Tripsacum. Note the pro-embryo at the top. Observations in other plants identify both intact synergids and unfused polar nuclei (not shown) (K) Developing seed in the same materials 4 DAP. The coenocytic endosperm is forming, but embryo development has not reinitiated yet. (L) Section of 7-DAP seed: a unique embryo forms, similar in apomictic and sexual plants. (M and N) Multiple embryos (arrows) within unique embryo sacs in Tripsacum with a single endosperm. (O) AFLP fingerprinting of two pairs (lanes 2 + 3 and 4 + 5) of twin embryos from the same mother plant (lane 1). Note that lanes 2 and 3 represent twins that are clones of the mother plant, while lanes 4 and 5 represent twins that are not identical to their mother plant and, hence, likely of sexual origin. Bar, 50 um.

from mitotic-like, rather than meiotic, first divisions. It is unclear whether those cells developed from a disrupted meiosis I or from a true meiosis II. Nevertheless, we never observed tetrads in our sampling. This strongly suggests that the dyads corresponded to the final stage of sporogenesis and therefore that they occurred from a meiosis II-like division, rather than from a disrupted meiosis I. It is also unclear on the basis of our observations whether one of the two cells degenerated or whether both cells progressed to megagametogenesis.



However, the frequency of multiple embryo sacs in mature ovules is extremely low, suggesting that a single functional spore was selected at the end of megasporogenesis (see next section).

Kernel development in sexual and apomictic Tripsacum: Mature unpollinated ovaries collected from sexual Tripsacum individuals showed seven-celled megagametophytes of the Polygonum type. After pollination, both the embryo and the endosperm followed developmental courses typical of those observed in maize. Most unpollinated ovaries collected at maturity from Tripsacum plants and apomictic maize-Tripsacum hybrids showed a single Polygonum-type megagametophyte. However, a major difference was the presence of globular pro-embryos in 26 out of 30 preparations that we observed (Figure 2). Most globular pro-embryos were located at the micropylar end between the central cell with unfused neighboring polar nuclei and intact synergid cells.



Presence of the synergids and the central cell nuclei shows that the pro-embryos developed from the egg cell. Pro-embryos contained 8–32 cells, indicating that the embryo had completed up to five mitotic divisions. Concomitant embryonic development was also observed in other cell types of the megagametophytes such as antipodal or synergid cells (two and three megagametophytes, respectively). The four remaining unfertilized megagametophytes showed a typical Polygonum-type organization with a normal-appearing egg cell.

Further development of the megagametophytes was observed at various times with and without pollination. In the later case, no development was noted in both pro-embryos and central cells, and the megagametophytes finally collapsed. Discharge of the pollen tube into megagametophytes collected from pollinated flowers was observed 8 hr after pollination (HAP). Further endosperm development followed a course similar to that observed in reduced megagametophytes. The first division of the primary endosperm nucleus was noted 20 HAP and resulted in a coenocytic sac with nuclei having migrated peripherally 3 DAP (Figure 2). After a short lag period, coenocytic endosperms started to cellularize between 4 and 5 DAP.

Although the pro-embryos were formed prior to pollination, they appeared arrested in development for several DAP. Throughout a 5-DAP period, they showed signs of neither further cell division nor developmental differentiation. First evidences of reinitiation of embryogenic development were noted 5 days after pollination as a few embryos at the transition stage were observed. At 7 and 8 DAP, embryos had reinitiated development in the 35 megagametophytes we observed (Figure 2). No evidences for a similar resting stage were found in sexual Tripsacum.

Polyembryos associated with single endosperms were observed in four cases (Figure 2). Polyembryony was further characterized by determining DNA content and fingerprints of embryos obtained from 157 polyembryonic kernels (Figure 2). Most pairs (129 or 82%) were composed of maternal clones. Among the remaining ones, we found mixtures of maternal and nonmaternal embryos (19 or 12%) and pairs of genetically identical but nonmaternal embryos (9 or 6%).

Male meiosis in Tripsacum: We estimated the proportion of reduced, aneuploid, and unreduced male gametophytes in both the sexual and apomictic accessions using flow cytometry. In both sexual diploid maize and sexual diploid Tripsacum samples, only reduced male gametophytes were detected using flow cytometry. Similarly, unreduced pollen grains or pollen grains with more than a $4x$ complement for meiotic products were not detected with flow cytometry in sexual tetraploid maize. In apomictic Tripsacum accessions, however, unreduced pollen grains or aneuploid pollen grains of high ploidy levels (close to and eventually higher than $4x$) represented on average 25% of the mature male gametophyte. Since a lot of the aneuploid products likely did not reach maturity because of unbalanced chromosomal complements, this estimate probably undervalues the frequency of abnormal male meiosis in apomictic samples.

To further characterize pollen development in apomicts, we analyzed male meiosis in four different apomictic accessions, including accession 65-1234. At least 200 cells covering all meiotic stages were scored for each entry. Results were consistent in all four accessions. Microsporogenesis in apomictic Tripsacum is characterized by numerous abnormalities (Figure 3). The first and foremost peculiarity concerns the synchronization of meiocyte development within the anthers; while, in sexual maize and sexual Tripsacum, meiocytes within an anther are usually well synchronized, this is not the case in apomictic Tripsacum. During meiosis I, for example, cells could be found from leptotene to pachytene in a single anther.

Wild-type meiocytes, *i.e.*, without noticeable defaults, were seldom observed (Table 2). Loose condensation of chromosomes was observed in ~12% of the cells from diakinesis to anaphase. Most cells at metaphase (>80%)

Figure 3.—Male meiosis in apomictic Tripsacum. (A–F) Abnormal male meiocyte development in apomictic Tripsacum. (A) DAPI staining of male meiocytes showing abnormal alignments at the metaphase I plate. (B) Normal organization of the meiotic spindle at metaphase I: the chromosomes, stained with propidium iodide, are shown in red, and the microtubules, stained with an antibody against tubulin, are shown in green. (C) Heterochronic cell division at metaphase I: cell division is near completion at a time when the chromosomes are still aligned in metaphase I. Note that the division is not only heterochronic but also misoriented at 90° from the expected division plane. (D) Misorientation of meiosis II: both cells in the dyad acquire opposite polarity. (E) A tetrad resulting from a misoriented meiosis II: the nuclei are stained with DAPI, and cell walls are visible thanks to a strong autofluorescence captured with Cy3 filters. (F) Uncoupling of cell division and cytokinesis during meiosis: the cells on the right result from an abnormal meiosis II in which one of the dyads divided without nuclear division, following a normal meiosis I; the cells on the left result from an abnormal meiosis I without nuclear division, followed by a normal meiosis II. (G–L) Pattern of histone H3 phosphorylation during meiosis in sexual (G and H) and apomictic (I–L) Tripsacum meiocytes. (G and H) Phosphorylation of histone H3 in sexual Tripsacum is similar to maize; no signals are observed before the end of diakinesis apart from the nucleolar organizing region. (I and J) Precocious phosphorylation in apomictic forms: the first signals are visible by the zygotene, with a limited number of initiation sites; note that not all chromosomes show initiation sites. (K) High levels of phosphorylation at diakinesis in apomicts. (L) Phosphorylation signals at metaphase are similar in sexual and apomictic plants and cover the entire chromosome arms. Bar, 25 um.

TABLE 2

Cytological analysis of male meiosis in apomictic Tripsacum accessions

Phenotype/stage	% of meiocytes
Loose chromosome condensation: prophase I	12 (238)
Early histone H3 phosphorylation: prophase I	55 (112)
Univalents: metaphase I	82 (243)
Heterochronic cell division: meiosis I	8 (134)
Orientation of cell division: meiosis II	25 (110)
Heterochronic cell division: meiosis II	7 (110)
Unreduced or aneuploid; mature pollen grain (a)	25 (b)

Frequency of abnormal phenotypes in meiocytes from five Tripsacum accessions. The data represent the sum of the five accessions. Samples size varies among stages (see text) and is indicated in parentheses. The data regarding the timing of histone H3 phosphorylation were obtained from a single accession (65-1234). (a) Measured using flow cytometry; (b) cell count in all samples were 5000 nuclei with five replicates.

contained both univalents and chromosomes that failed to align to the metaphase plate (Figure 3, A and B). They still formed spindles that were similar to the wild-type meiocytes. Most of the unaligned chromosomes were not attached to the main spindle. Some of them created a local array of disorganized microtubules. In 8% of the meiocytes at metaphase, a marked asynchrony between chromosome behavior and cell division was observed. In those cells, cytokinesis took place during the meiotic prophase I. As observed in Figure 3C, cell division is near completion while the chromosomes are still aligned at the metaphase plate, and the meiotic spindle is fully formed. Defaults in cell division were also observed at later stages (Figure 3, D–F). In 32% of the meiocytes in meiosis II, cytokinesis was either independent of cell division (taking place before nuclear division) or misoriented.

Although virtually all cells reveal some defaults, there is no consistency in phenotype; individual cells might or might not present one or more of the above characteristics. Rather, male meiosis is apparently disturbed in numerous facultative ways, resulting in an array of possible phenotypes.

Pattern of histone phosphorylation during male meiosis: An antibody that recognizes a ser10 phosphoepitope on histone H3 was used to monitor H3 phosphorylation during meiosis in both sexual and apomictic materials. Histone H3 phosphorylation has been reported to be an excellent marker of condensation for meiotic and mitotic chromosomes in maize (Kaszas and Cande 2000). In mitotic cells, histone H3 phosphorylation starts during late prophase and reaches its maximum at metaphase. At metaphase, only pericentromeric regions are phosphorylated, with little or no phosphorylation along the arms. In meiotic cells, histone H3 becomes phosphorylated just prior to metaphase. Phosphorylation starts in the pericentromeric regions and later extends through the arms at metaphase I. When chromosomes are fully condensed at metaphase I, they are uniformly stained. At metaphase II, by contrast, only the pericentromeric regions are stained, reflecting mitotic-like chromosome morphology.

We scored a minimum of 100 cells per entry at each stage mentioned hereafter. In both sexual maize and sexual Tripsacum, patterns of histone H3 (Figure 3, G and H) phosphorylation conformed to the published literature (Kaszas and Cande 2000). Patterns of H3 phosphorylation were more irregular in apomictic materials and differed from cells to cell (Figure 3, I–L). In ~55% of the male meiocytes in accession 65-1234, H3 phosphorylation was first observed before or during pachytene (Table 2). In those cells, it started with 8–13 initiation sites (average 11, calculated on the basis of 58 cells with clear signals at pachytene), the remaining chromosomes showing no phosphorylation signals. By diakinesis, >70% of the cells showed phosphorylation signals. After diakinesis, normal phosphorylation patterns were observed in all cells.

DISCUSSION

Apomeiosis results from heterochronic termination of female meiosis: In sexual Tripsacum as in sexual maize plants, ovule development and female gamete formation are synchronized; ovules at a particular time in organogenesis contain reproductive cells at predictable stages. Thus, meiocyte development in sexual and apomictic plants can be compared for similar developmental steps by monitoring ovule development. At stages of ovule development corresponding to early meiosis in sexual Tripsacum and maize plants, meiotic chromosomes were never observed in apomictic accessions. Instead, meiocytes were either in premeiotic interphase or already differentiated as immature uninucleate embryo sacs. These observations are consistent with the expected Antenaria type of diplospory in Tripsacum: the MMC totally skips meiosis and directly differentiates into a uninuclear embryo sac. The entire set of events taking place during wild-type sporogenesis is short circuited.

In contrast to apomictic Tripsacum, the modalities of apomeiosis in the 38C maize-Tripsacum derivatives varied significantly from cell to cell. The reasons for such plasticity are unclear. Nevertheless, our observations provide valuable details regarding the mechanisms of diplospory. Early stages of meiosis I, leptotene to pachytene, were observed in the 38C plants. However, the occurrence of meiosis in 38C was rare (<2% of the progeny). Because our analysis techniques are destructive, we could not observe the various stages of apomictic

development on the same ear. Nonetheless, there is no evidence to indicate that the subsets that were used to evaluate early diplosporous stages and progeny types differed in any respect. Since our survey of the progeny types in 38C materials was almost exhaustive (94% of the flowers sampled), we assume that most of the cells that initiated meiosis produced unreduced gametes. A likely explanation can be found in cells observed later during development. Cells predicted in metaphase I on the basis of ovule development could be classified into two groups. The first one includes multinucleate embryo sacs. These likely arose from an Antenaria type of development. The second group, with a proportion similar to the cells initiating meiosis at an earlier stage (26%), includes cells in various mitotic configurations.

From those observations, we understand that most meiocytes initiating meiosis neither completed the process (reduced spores represent <2%) nor aborted (98% of the flowers produce unreduced egg cells). Rather, we propose that those cells revert to mitosis, thereby overturning their earlier commitment to meiosis. Under this hypothesis, diplospory in Tripsacum would affect early steps of megaspore formation, inducing a heterochronic exit from meiosis. In the extreme heterochronic phenotype, meiosis would be skipped entirely.

Similar processes have been reported in yeast: early meiotic cells can reenter a mitotic cell cycle when transferred from sporulation to growth medium (Honigberg and Esposito 1994; McCarroll and Esposito 1994). In yeast, pachytene represents the latest stage at which meiotic cells can be forced to reenter a mitotic cell cycle. Since the latest meiotic stages observed in our materials were early pachytene, we assume that apomeiosis occurs by terminating meiosis any time between MMC differentiation and pachytene. The occurrence of rare events of complete meiosis in Tripsacum indicates that, similar to yeast, there might be a limit after which the commitment to meiosis becomes irreversible. Our data do not allow us to define precisely this limit. Nevertheless, the sharp reduction in the frequency of meiotic cells between early meiotic stages (stage 1 in Table 1) and late prophase I (stage 2 in Table 1) suggests that this limit occurs during early prophase I, likely before completion of pachytene.

We cannot speculate on the mechanisms inducing an exit from meiosis. However, our observations suggest that diplospory is not the result of altered meiotic functions. In particular, diplosporous plants conserved intact the potential for both male and female meiosis. Furthermore, diplospory in Tripsacum leads to the formation of functional gametes. Meiosis, however, is actively controlled by checkpoints (Roeder and Bailis 2000), whose function is to ensure that future events do not occur before previous events have been successfully completed. The production of functional spores and embryos indicates an absence of checkpoints on the outcome of diplospory. This suggests that the processes themselves are essentially "wild type." Thus, we propose that the diplosporous phenotype affects the developmental timing of sporogenesis, but not the core functions required for sporogenesis *per se*.

According to a popular model, the early termination of sporogenesis results from an early induction of gametogenesis. Our observations point to a different model. In particular, we observed that the dyads observed at stage 2 (see Table 1) resulted from mitotic-like, rather than meiotic, first divisions. This indicates that the cells that failed the first meiotic division remained committed to sporogenesis, rather than to gametogenesis. Thus, termination of meiosis occurred independently of the initiation of gametogenesis. Altogether we therefore conclude that diplospory induced heterochronic exit from meiosis by affecting the developmental timing of sporogenesis.

Our observations also suggest an important difference between the aposporous and diplosporous types of development. In aposporous apomictic plants, a somatic cell from the nucellus differentiates into a spore and undergoes the postmeiotic events of gametogenesis. This implies that the first consequence of apospory is a shift in cell fate within the nucellus (Tucker *et al.* 2003). Our observations suggest that diplosporous MMCs do not undergo the same shift: the MMCs differentiate similarly in diplosporous and sexual plants, and the potential for female meiosis remains unaltered. This suggests that apospory and diplospory probably rely on distinct mechanisms. In many ways, diplospory and apospory mimic the differences observed between heterochronic and homeotic mutants: while the former alters cell fate within temporal domains (and is thus heterochronic), the latter alters cell fate within spatial domains, similar to homeotic mutants.

Parthenogenesis results from heterochronic induction of early embryogenesis: In sexual plants, seed development relies upon double fertilization. Here, we show that in apomictic Tripsacum, pro-embryos progress up to five divisions prior to fertilization. The presence of pro-embryos in Tripsacum has been reported previously (Farquharson 1955; Bantin *et al.* 2001). A significant new finding reported here is an arrest of embryo growth after three to five divisions, which resumes only after endosperm cellularization.

This observation suggests that the embryo in apomicts passes through two clearly different stages of development, separated by a crucial transition point and taking place after three to five divisions. The first stage, represented by the pro-embryo, is independent of fertilization. Rather, pro-embryo development is part of the formation of the mature embryo sac. It is therefore fully under maternal control.

Recent reports have shown that early seed development in Arabidopsis is largely under maternal control and that most male-derived alleles are silent during the first divisions of the zygote (Vielle-Calzada *et al.* 2000).

Although the data are still controversial (Springer *et al.* 2000; Baroux *et al.* 2001; Weijers *et al.* 2001), the Vielle-Calzada observations come as no surprise when considering apomictic plants: the formation of apomictic embryos involves no paternal genome, indicating that the male contribution is clearly dispensable. Interestingly, the timing of reinitiation of paternal transcription in Arabidopsis is similar to the timing of reinitiation of embryo development in apomicts. This again suggests that an essential transition, which corresponds to the end of the maternally controlled gametophytic phase, takes place similarly in both the apomictic and sexual plants after roughly five divisions. Apomixis, from that perspective, affects only the mechanisms and timing of early embryo development.

What induces precocious embryo development in apomicts is unclear. It is often mentioned in the literature that parthenogenesis might be a pleiotropic consequence of apomeiosis (Nogler 1984; Grimanelli *et al.* 2001; Grossniklaus *et al.* 2001). Our analysis of polyembryonic seeds questions such a hypothesis. The information from 157 polyembryonic kernels indicates multiple origins, including several cells from an unreduced embryo sac, cells from multiple embryo sacs (*e.g.*, mixtures of maternal and nonmaternal embryos), and also, significantly, from different cells in a single reduced embryo sac (pairs of genetically identical but nonmaternal embryos). The latter in particular indicate that pro-embryos can form irrespective of diplospory, suggesting no functional relationship. This is in agreement with observations made in other apomictic systems, such as *Erigeron annuus*, where apomeiosis and parthenogenesis are genetically unlinked traits (Noyes and Rieseberg 2000). From these considerations, we conclude that diplospory is not pleiotropic to parthenogenesis in Tripsacum. Rather, both processes reflect a more global alteration of reproductive pathways affecting sporogenesis and early embryogenesis.

Male meiosis reveals unique aspects of apomixis in Tripsacum: It is generally accepted that diplospory is a female-specific trait. Here, however, we showed that diplospory likely affects early stages of female meiosis. Furthermore, our observations also suggest that in Tripsacum, the progression of both male and female meiosis is affected in similar ways. As such, microsporogenesis, which is much more exploitable for cellular characterization, might provide a useful source of indirect information regarding the cellular processes involved in apomeiosis.

Flow cytometry indicates that meiosis in four distinct apomictic Tripsacum accessions results in a large proportion of unreduced or aneuploid male gametophytes, together with reduced pollen grains. Although virtually all male meiocytes in apomictic Tripsacum present abnormal development, we could not identify any specific phenotype. Rather, male meiosis is seemingly altered in numerous, facultative ways. In all instances, those alterations relate to the timing of the initiation of cellular events. Not all the phenotypic abnormalities observed in male meiocytes are necessarily a primary effect of diplospory. Apomixis plants can certainly accumulate mutations in the male function without significantly altering their overall fitness. Nevertheless, the defects reported here are unlikely to result exclusively from accumulation of unrelated mutations. A notable feature of male meiosis in apomictic Tripsacum is the absence of clear-cut phenotypes. This reflects a relaxed selection of the progression of meiosis rather than altered meiotic functions, a phenomenon that is a hallmark of female sporogenesis in apomictic Tripsacum. Three processes are particularly illustrative. The first one is the loss of synchronization between cells within the anthers. The second one is the loss of synchronization between cell division and chromosome movements within individual meiocytes. This occurs at several stages, including metaphase I and prophase II, and shows that, in apomictic Tripsacum, developmental programs (such as cytokinesis and nuclear division) can be superimposed on each other, leading to the precocious termination of the earlier of the two programs. This is very similar to what was observed for the termination of meiosis in female meiocytes in apomicts.

The third process is the pattern of histone H3 phosphorylation. In apomicts, histone H3 phosphorylation occurs much earlier than in sexual materials, illustrating the heterochronic expression of pieces of the meiotic process. Interestingly, not all chromosomes show synchronized phosphorylation. The number of initiation sites is limited to a subset of the chromosomes, an observation unique to apomicts. This supports the "genome asynchrony" model for the regulation of apomixis (Carman 1997). Under this hypothesis, allopolyploids with divergent genotypes could cause asynchrony in the expression of the regulatory genes that control reproductive programs. This would lead to the concurrent asynchronous expression of unaltered developmental programs and ultimately to apomeiosis. The immunochemistry data presented here indicate that not all chromosomes in polyploid apomictic Tripsacum condense at the same time. Although we cannot demonstrate that the same chromosomes are affected across meioses, it will be interesting to verify whether there might be divergence for developmental signals among the genomes that comprise apomictic Tripsacum.

Conclusion: apomixis results from a global deregulation of sexual developmental programs: During sexual development, reproductive organs and cells within the reproductive organs undergo predictable and synchronized temporal and spatial changes. Not so in apomictic Tripsacum, where the progression through spore, gamete, and embryo formation suffers alterations as compared to the sexual forms. Our current model is summarized in Figure 4. Our data indicate that apomixis in Tripsacum causes a highly plastic heterochronic pheno-

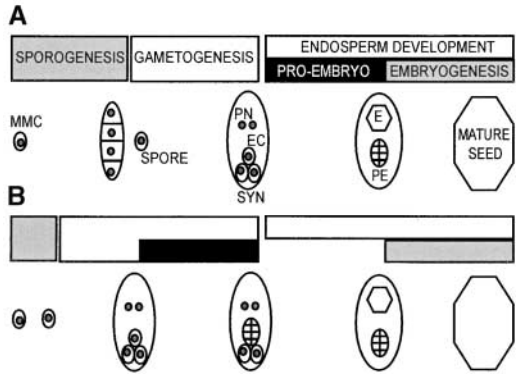


FIGURE 4.—Relative developmental timing of sporogenesis and pro-embryo development in sexual (A) and diplosporous apomictic Tripsacum (B). In this model, we assume (see text) that the main alterations to the progression of the sexual pathway affect sporogenesis (via an heterochronic exit from meiosis) and the formation of a pro-embryo (via heterochronic development). Sporogenesis is shortened in the diplosporous pathway; in the extreme heterochronic phenotype, it is reduced to a shift of fate from MMC to the functional spore. Gametogenesis, endosperm development, and embryogenesis (past the globular stage) are essentially wild type. MMC, megaspore mother cell; PN, polar nuclei; SYN, synergids; EC, egg cell; E, endosperm; PE, pro-embryo. The size of the boxes is not proportional to the actual duration of the corresponding stages.

type, resulting from a deregulation of the developmental timing of sporogenesis and early embryogenesis. Apomixis in Tripsacum is built of blocks of the sexual development pathways, whose expression is altered in time. It depends on an alteration of the orderly progression of developmental subroutines, rather than on the establishment of a new function. How such a model might fit with the current genetic evidence, which implies a limited number of mutant genes, remains an open question. Genetic screens for regulators of the developmental timing of female reproduction might shed some light on this fascinating problem.

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LITERATURE CITED

ASKER, S., and L. JERLING, 1992 *Apomixis in Plants*. CRC Press, Boca Raton, FL.

BANTIN, J., F. MATZK and T. DRESSELHAUS, 2001 Tripsacum dactyloides (Poaceae): a natural model system to study parthenogenesis. Sex. Plant Reprod. **14:** 219–226.

BAROUX, C., R. BLANVILLAIN and P. GALLOIS, 2001 Paternally inherited transgenes are down-regulated but retain low activity during early embryogenesis in Arabidopsis. FEBS Lett. **509:** 11–16.

BASS, H. W., W. F. MARSHALL, J. W. SEDAT, D. A. AGARD and W. Z. CANDE, 1997 Telomeres cluster de novo before the initiation of synapsis: a three-dimensional spatial analysis of telomere positions before and during meiotic prophase. J. Cell Biol. **137:** 5–18.

CARMAN, J. G., 1997 Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. Biol. J. Linn. Soc. **61:** 51–94.

FARQUHARSON, L. I., 1955 Apomixis and polyembryony in Tripsacum dactyloides. Am. J. Bot. **42:** 737–743.

GOLUBOVSKAYA, I., N. A. AVALKINA and W. F. SHERIDAN, 1992 Effects of several meiotic mutations on female meiosis in maize. Dev. Genet. **18:** 411–424.

GRIMANELLI, D., O. LEBLANC, E. ESPINOSA, E. PEROTTI, D. GONZALEZ DE LEON *et al.*, 1998 Non-Mendelian transmission of apomixis in maize-Tripsacum hybrids caused by a transmission ratio distortion. Heredity **80:** 40–47.

GRIMANELLI, D., O. LEBLANC, E. PEROTTI and U. GROSSNIKLAUS, 2001 Developmental genetics of gametophytic apomixis. Trends Genet. **17:** 597–604.

GROSSNIKLAUS, U., G. A. NOGLER and P. J. VAN DIJK, 2001 How to avoid sex: the genetic control of gametophytic apomixis. Plant Cell **13:** 1491–1498.

HOISINGTON, D., M. KHAIRALLAH and D. GONZÁLEZ-DE-LEÓN, 1994 *Laboratory Protocols: CIMMYT Applied Molecular Genetics Laboratory*, Ed. 2. CIMMYT, México D. F., México.

HONIGBERG, S. M., and R. E. ESPOSITO, 1994 Reversal of cell determination in yeast meiosis: postcommitment arrest allows return to mitotic growth. Proc. Natl. Acad. Sci. USA **91:** 6559–6563.

HUANG, B.-Q., and W. F. SHERIDAN, 1996 Embryo sac development in the maize indeterminate gametophyte1 mutant: abnormal nuclear behavior and defective microtubule organization. Plant Cell **8:** 1391–1407.

KASZAS, E., and W. Z. CANDE, 2000 Phosphorylation of histone H3 is correlated with changes in the maintenance of sister chromatid cohesion during meiosis in maize, rather than the condensation of the chromatin. J. Cell Sci. **113:** 3217–3226.

KOLTUNOW, A. M., 1993 Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. Plant Cell **5:** 1425–1437.

LEBLANC, O., M. D. PEEL, J. G. CARMAN and Y. SAVIDAN, 1995 Megasporogenesis and megagametogenesis in several Tripsacum species (Poaceae). Am. J. Bot. **82:** 57–63.

LEBLANC, O., D. GRIMANELLI, N. ISLAM-FARIDI, J. BERTHAUD and Y. SAVIDAN, 1996 Reproductive behavior in maize-Tripsacum polyhaploid plants: implications for the transfer of apomixis into maize. J. Hered. **87:** 108–111.

MCCARROLL, R. M., and R. E. ESPOSITO, 1994 SPO13 negatively regulates the progression of mitotic and meiotic nuclear division in *Saccharomyces cerevisiae*. Genetics **138:** 47–60.

NOGLER, G. A., 1984 Gametophytic apomixis, pp. 475–518 in *Embryology of Angiosperms*, edited by B. M. JOHRI. Springer-Verlag, Berlin.

NOYES, R. D., and L. H. RIESEBERG, 2000 Two independent loci control agamospermy (apomixis) in the triploid flowering plant *Erigeron annuus*. Genetics **155:** 379–390.

ROEDER, G. S., and J. M. BAILIS, 2000 The pachytene checkpoint. Trends Genet. **16:** 395–403.

RUSSELL, S. D., 1978 Fine structure of megagametophyte development in *Zea mays*. Can. J. Bot. **57:** 1093–1110.

SAVIDAN, Y., 2000 Apomixis: genetics and breeding, pp. 13–86 in *Plant Breeding Reviews*, edited by J. JANICK. John Wiley & Sons, New York.

SPRINGER, P. S., D. R. HOLDING, A. GROOVER, C. YORDAN and R. A. MARTIENSSEN, 2000 The essential Mcm7 protein PROLIFERA is localized to the nucleus of dividing cells during the G(1) phase and is required maternally for early Arabidopsis development. Development **127:** 1815–1822.

TUCKER, M. R., A. C. ARAUJO, N. A. PAECH, V. HECHT, E. D. SCHMIDT *et al.*, 2003 Sexual and apomictic reproduction in Hieracium subgenus Pilosella are closely interrelated developmental pathways. Plant Cell **15:** 1524–1537.

VIELLE-CALZADA, J. P., R. BASKAR and U. GROSSNIKLAUS, 2000 Delayed activation of the paternal genome during seed development. Nature **404:** 91–94.

WEIJERS, D., N. GELDNER, R. OFFRINGA and G. JURGENS, 2001 Seed development: early paternal gene activity in Arabidopsis. Nature **414:** 709–710.

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