Reproductive Behavior in Maize-*Tripsacum* Polyhaploid Plants: Implications for the Transfer of Apomixis Into Maize

O. Leblanc, D. Grimanelli, N. Islam-Faridi, J. Berthaud, and Y. Savidan

Expression of gametophytic apomixis in grasses is restricted to polyploid plants. In our effort to transfer apomixis from diplosporous tetraploid *Tripsacum* ($2n = 4x = 72$) into maize ($2n = 20$) through conventional backcrosses, we produced polyhaploid plants combining one complete set of chromosomes from each genus. These polyhaploid plants were totally male sterile. By contrast, viable seeds were produced apomictically when they were pollinated using maize. Apomictic reproduction in such polyhaploids, which show a diploid-like chromosomal complement, suggests that diplosporous apomixis and polyploidy are not totally linked and that diploid crops such as maize might reproduce apomictically.

Gametophytic apomixis, an asexual reproduction through seeds (Nogler 1984a), commonly occurs in Angiosperms such as wild grasses and Asteraceae (Asker and Jerling 1992). Its introduction into crops would open new areas in plant breeding and agriculture, especially in Third World countries (Savidan and Dujardin 1992). In agamic complexes, diploids are typically sexual, whereas polyploids reproduce apomictically (Stebbins 1950). Most host crop species, however, are diploid. Obstacles to the transfer of apomixis through conventional backcrossing from wild-related species into crops are numerous (e.g., genomically distant apomictic donor to the host species, male sterility in wide hybrids, detection of apomixis in wide hybrids and backcross progeny), but this strong relationship between gametophytic apomixis and polyploidy represents one of the most important.

From diplosporous tetraploid *Tripsacum*, the closest wild apomictic relative of maize ($2n = 20$) with $x = 18$ (Harlan et al. 1970; Reeves and Mangelsdorf 1935), we established a collection of several generations of maize-*Tripsacum* hybrids. From apomictic $2n = 56$ hybrids (i.e., 20 maize chromosomes + 36 from the wild species), we produced apomictic polyhaploid plants ($2n = 28$) combining one haploid set of chromosomes from each maize and *Tripsacum*. Although apomictic polyhaploid plants have been already reported in aposporous agamic complexes, they were generally weak and sterile (Nogler 1984a). De Wet et al. (1973), however, already mentioned fertile 28-chromosome plants derivating from maize-*Tripsacum* hybrids, but neither mode of reproduction nor chromosomal complement were clearly documented.

The aims of this study were to document the chromosome complement and the reproductive behavior in one of these maize-*Tripsacum* polyhaploids from a research program designed to move gene(s) responsible for diplosporous apomixis into maize. Possibilities of expression of apomixis in diploid maize plants are also discussed.

Materials and Methods

Plant Material

One 28-chromosome polyhaploid plant, 6-529, was obtained through a complex backcrossing scheme from F_{1-16}, a maize-*Tripsacum* F_1 hybrid. F_{1-16} was $2n = 46$—i.e., 36 chromosomes from *Tripsacum* (Tr) and 10 from maize (M). It was obtained using a maize plant ($2n = 20$) from CIMMYT population 34 as the pistillate parent and CIMMYT *Tripsacum dactyloides* accession 65-1234, an apomictic tetraploid ($2n = 72$) from Evergleades (Florida, U.S.A.), as the male parent. Because of complete male sterility in F_{1-16} and its backcross derivatives, plant 6-529 was derived from F_{1-16} after six generations of backcross using pollen from maize individuals from CIMMYT population 21 (Table 1A). 6-529 was then pollinated with a maize hybrid (CML62 × CML135, CIMMYT lines), resulting in a progeny of 64 plants.

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Table 1. Maize-*Tripsacum dactyloides* 65-1234 hybrid generations from which $2n = 28$ polyhaploid plant, 6-529, originated

		Generations of backcrosses					
	F_1	G_1	G_2	G_3	G_4	G_5	G_6
A							
Plant no.	16	1,001	2,017	3,031	4,031	5-8	6-529
No. of chromosomes	$2n = 46$	$2n = 56$	$2n = 56$	$2n = 56$	$2n = 56$	$2n = 56$	$2n = 28$
Origin		$2n + n$	$2n + 0$	$2n + 0$	$2n + 0$	$2n + 0$	$n + 0$
B							
$2n = 46$ (36Tr + 10M)	6	—	—	—	—	—	—
$2n = 56$ (36Tr + 20M)	1	15	9	1	29	1	—
$2n = 66$ (36Tr + 30M)	—	—	—	—	6	—	—
$2n = 28$ (18Tr + 10M)	—	—	—	—	—	1	—

Hybrids were backcrossed using maize pollen ($n = 10$). A: Chromosome number and origin of each genotype involved in the production of plant 6-529. B: Chromosome numbers detected in the progeny of each genotype involved in the production of plant 6-529. Tr = *Tripsacum* chromosomes; M = maize chromosomes.

Chromosomal Analyses

Chromosome numbers of F_{1-16} and derivatives in the five first backcross generations were determined from root tips (prefixed at 4°C in saturated 8-hydroxyquinoline for 4 h, fixed in Carnoy's solution for 24 h, and stored at −4°C in 70% ethanol) squashed and observed by phase-contrast microscopy. Flow cytometry was used to estimate the chromosome numbers in plant 6-529 and its 64 offspring. Leaf samples were prepared according to Galbraith et al. (1981) and analyzed with a PARTEC CA-II cytometer (Partec GmbH, Münster, Germany). We also performed fluorescing in situ hybridization to determine the chromosomal complement of plant 6-529 and four offspring (three morphologically similar to the mother plant and one morphologically different). Chromosome preparations were made following Jewell and Islam-Faridi (1994), and DNA labeling and detection procedures according to Islam-Faridi and Mujeeb-Kazi (1995).

Determining Mode of Reproduction

Megasporogenesis analyses. Callose deposition patterns throughout megasporogenesis are reliable indicators of the mode of reproduction in *Tripsacum* spp. (Leblanc et al. 1995b): callose synthesis, that is observed in and around cell walls of meiotic megasporocytes and their derived megaspores, does not occur during diplosporous development. For callose analyses, 30 pistils from 6-529 and 15–25 from 10 offspring (eight morphologically similar to 6-529 and two morphologically different, including the four previously analyzed by in situ hybridization) were first optically cleared using three sucrose solutions (200, 500, and 800 g/L, respectively) supplemented with aniline blue (100 mg/L), and examined using ultraviolet vertical epifluorescence microscopy (Leblanc et al. 1995b). In addition, to confirm callose analyses, pistils were recleared for interference contrast microscopy using a benzyl benzoate-dibutyl phthalate procedure (Crane and Carman 1987) for internal detail observations within ovules.

Fingerprinting analyses for 6-529 progeny testing. Total genomic DNA was extracted according to Hoisington et al. (1994) from the *Tripsacum* parent 65-1234, plant 6-529, and 13 offspring (10 morphologically similar to 6-529 and three morphologically different, including the four previously analyzed by in situ hybridization). Ten micrograms of DNA were digested with restriction endonuclease *HindIII,* electrophoresed, and transferred onto nylon membrane. Hybridizations, stringency washes, detection, and exposure were made according to Hoisington et al. (1994). We chose maize probes UMC3, UMC8, UMC38, UMC 310, and BNL16-06 because they were known to give multiple band patterns from a previous survey of an ORSTOM-CIMMYT *Tripsacum* collection (unpublished data).

Results

Chromosome numbers and morphology in progenies of plant F_{1-16} and derivatives, 1001, 2017, 3031, 4031, and 5-8, indicated that they reproduced through facultative apomixis (Table 1B). Two types of progenies were produced: maternal types ($2n + 0$) that showed both chromosome number and morphology similar to the mother plant, and offtypes that differed in morphology and chromosome number from the mother plant. These offtypes were classified (a) as $2n + n$ offtypes when chromosome numbers corresponded to the fertilization of an unreduced femelle gamete (i.e., $2n = 56 = 36Tr + 20M$ individual in F_{1-16} progeny, $2n = 66 = 36Tr + 30M$ individuals in 4031 progeny), or (b) as polyhaploid ($n + 0$) when they corresponded to the parthenogenetic development of a reduced gamete (i.e., plant 6-529 from 5-8 progeny).

Chromosomal Analyses

Plant 6-529 and 52 of its offspring were estimated to have $2n = 28$ using flow cytometry. The 12 remaining plants were estimated to have $2n = 38$. In situ hybridization in plant 6-529 and the 28-chromosome plants from its progeny we analyzed revealed that all these hybrids combined 10 and 18 chromosomes respectively from maize and *Tripsacum* (Figure 1a). The 38-chromosome offspring showed a 20M + 18Tr complement (Figure 1b).

This confirmed that 28-chromosome plants combine one complete haploid set of chromosomes from *Tripsacum* 65-1234 and one from maize, and therefore have a polyhaploid structure similar to that of a diploid.

Morphology and Fertility

6-529 and its 28-chromosome offspring were morphologically uniform and characterized by a particular phenotype resembling *Tripsacum* 65-1234 but quite distinct from accessions of the maize-*Tripsacum* hybrid collection derived from it: plant height was shorter, leaves narrower, and inflorescences shorter, carrying both fertile pistillate and atrophied staminate spikelets. 38-chromosome plants strongly differed from the wild type by recovering several traits from the crop: solid single stem generally with one or more weak tillers, leaves borne alternately on either side of the stem at each node, and male and femal spikelets borne in separate inflorescences. All 28- and 38-chromosome plants were male-sterile. Anthers were not dehiscent and pollen completely aborted, possibly as a result of meiotic irregularities due to the presence of two nonhomologous genomes. In contrast, viable seeds were produced when the plants were pollinated with maize pollen.

Mode of Reproduction

Megasporogenesis in 6-529 and in its 28- and 38-chromosome offspring was characterized by the complete failure of meiosis, as revealed by callose analyses and interference-contrast observations. Among all the pistils analyzed, no callose was detected during megasporogenesis, and enlarged megasporocytes developed directly into female gametophytes through mitoses. This reproductive pathway is similar to the diplosporous development observed in *T. dactyloides* 65-1234, the apomictic parent from

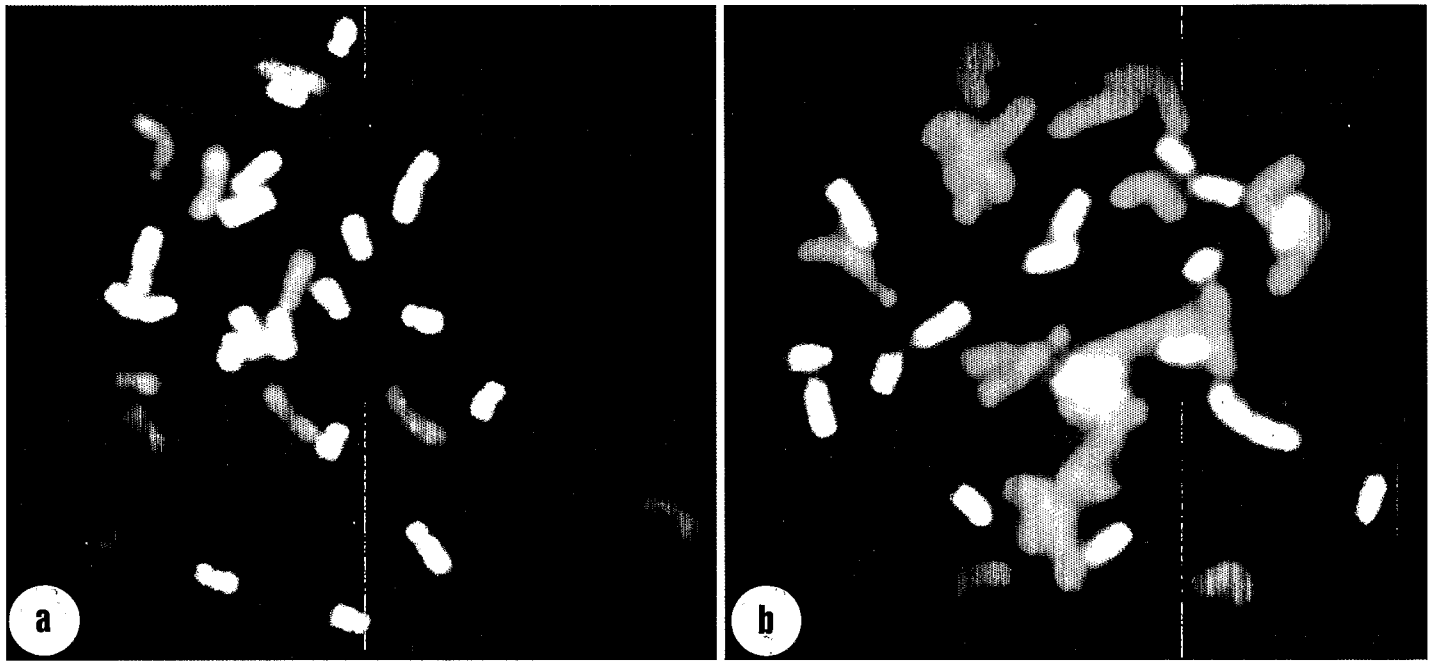

Figure 1. Root tip metaphases from 28- and 38-chromosome maize-*Tripsacum* hybrids after genomic fluorescing in situ hybridization, using total genomic DNA from *T. dactyloides* 65-1234 as a probe (yellow-labeled chromosomes are from the wild species, while the red-labeled ones are from maize). **(a)** Diploid-like structure in 28-chromosome polyhaploid hybrids that combine 10 maize chromosomes and 18 from the wild species. **(b)** A 38-chromosome plant, combining one haploid chromosome set from *Tripsacum* and the complete chromosome stock from maize.

which these plants were derived (Leblanc et al. 1995b).

All 28-chromosome plants from 6-529 progeny produced the same restriction fragment patterns as the mother plant when hybridized with the five selected maize probes, suggesting that neither recombination nor egg cell fertilization had occurred. Plants with 38 chromosomes showed additional bands which probably originated from the maize pollen thus confirming fertilization of some of the unreduced gametes of 6-529 ($2n + n$ offspring).

$2n = 28$ polyhaploids have been also found at a low frequency (<0.2%) in progeny of other BC1 plants derived from 65-1234. Those investigated for mode of reproduction also reproduced apomictically as revealed by progeny tests based on both chromosome numbers and morphology (at least 25 offsprings per mother plant) and by cytoembryological analyses (at least 15 pistils per mother plant).

Discussion

28-chromosome polyhaploid plant 6-529 originated through parthenogenetic development of a reduced gamete in 56-chromosome apomictic plant 5-8. Observations of megasporogenesis and results from progeny testing (based on morphology, chromosome numbers and molecular data) clearly indicated that the polyhaploid plant 6-529 also reproduced through facultative apomixis with the following patterns typical of apomictic reproduction (Bashaw 1980): (a) meiosis failure and expression of diplospory; (b) maternal offspring (81.5%) at the morphological and molecular levels; (c) presence of $2n + n$ offtypes (i.e., $2n = 20M + 18$ Tr) in a proportion (18.5%) commonly observed in apomictic maize-*Tripsacum* hybrids derived from plant 65-1234 (Leblanc 1995).

Pathways of genetic transfer from *Tripsacum* to maize through conventional backcrossing have been already described by Harlan and de Wet (1977). Maize plants possessing *Tripsacum* introgressions can be recovered in four steps from maize-*Tripsacum* BC1 hybrids ($2n = 56$) through sexual reproduction. However, because our goal is to transfer apomixis into maize and since male sterility strongly affects maize-*Tripsacum* F_1 hybrids and their 56-chromosomes derivatives, hybrids that contribute to the next step must be offtypes arising through residual sexuality. The frequency of such offtypes was estimated to be ~3% in *T. dactyloides* 65-1234 and its $2n = 56$ derivatives (Leblanc 1995). Putative apomictic addition lines that combine 20 chromosomes from maize and a few from *Tripsacum* will result from residual sexuality in apomictic 38-chromosome plants (18Tr + 20M). $2n = 38$ hybrids can be $n + n$ offtypes in progenies from $2n = 56$ hybrids, and are expected to segregate for mode of reproduction. Therefore, using this pathway (46 → 56 → 38 → addition lines) and given the low rate of residual sexuality in apomictic 56-chromosome hybrids, producing and identifying apomictic 38-chromosome hybrids requires the capacity to analyze a large number of progenies from $2n = 56$ plants. When backcrossed to maize, apomictic 28-chromosome polyhaploids are thus a good source of apomictic 38-chromosome plants, due to the frequency of $2n + n$ offtypes (~20%) in their progeny. This type of backcross is also of great interest for cytogenetic analyses: recovering a 20-chromosome apomictic plant from an apomictic maize-*Tripsacum* addition line would imply that exchanges of genetic material do occur at meiosis. The particular chromosome constitution of these polyhaploids appears to facilitate the study of the possible pairings between maize and *Tripsacum* chromosomes. Although pairings and exchanges between maize and *Tripsacum* have already been reported (Kindiger and Beckett 1990; Maguire 1960), the chromosome complement of the polyhaploids may allow researchers to determine whether preferential pairing exists and, if so, which chromosomes are involved.

In species that reproduce following the aposporous type of gametophytic apomix-

is, nonreduction is controlled by one dominant allele A (reviewed by Asker and Jerling 1992). Although very few data are available for diplosporous species, diplospory also appears to be simply inherited in *Eragrostis curvula* (Voigt and Bashaw 1972), and in *Tripsacum dactyloides* (Leblanc et al. 1995a). All models describing the evolution of apomixis show that, under this single-dominant-gene hypothesis, apomixis will become fixed unless it dramatically reduces the fitness of its carriers (Marshall and Brown 1981; Pernès 1972). Apomixis is usually not expressed at the diploid level in agamic complexes; but diploid genetic pools do make a large genetic contribution to such complexes by producing new variability through recombination and, at the same time, by exchanging genetic material with polyploids (de Wet 1968; Savidan and Pernès 1982). The strong interrelationship between apomixis expression and polyploidy in wild agamic complexes is not well understood yet, and mechanisms of protection against apomictic reproduction at the diploid level still have to be elucidated. Dihaploids obtained from apomicts in the *Bothriochloa-Dichanthium* complex (de Wet 1968) and in *Panicum maximum* (Savidan 1982) were either sexual and fertile or potentially apomictic, as aposporous embryo sacs were observed, but sterile. Genetic models such as the allelic ratio hypothesis, which postulates that sterility arises when the proportion of A to a alleles in a given individual is >0.25 explain sterility in dihaploids from the *Panicum maximum* complex (Noirot 1993); however, they do not fit the $2n = 28$ and 38 maize-*Tripsacum* hybrids described here, for which the allelic ratios are expected to be 0.5 and 0.33, respectively (the *Tripsacum* parental genotype is simplex for the allele conferring diplospory; Leblanc et al., 1995a). Besides, a few diploid species (largely from dicotyledonous taxa) reproduce apomictically (Asker and Jerling 1992), and apospory was also expressed in a trisomic hybrid form ($2n + 1$) of *Ranunculus* (Nogler 1984b).

The expression of apomixis in partially fertile polyhaploids from apomicts has been already reported in *Ranunculus argoviensis* (Nogler 1984a), and in two derivatives ($2n = 21$ and $2n = 23$) from interspecific hybrids between *Pennisetum glaucum* L. ($x = 7$) and wild *Pennisetum* species ($x = 9$) (Dujardin and Hanna 1986; Hanna et al. 1992). However, the origin of the chromosomes in these *Pennisetum* polyhaploids could not be determined. In regard to the genetic factors responsible for apomixis, this does not clearly indicate nonpolyploid conditions for their expression: both $2n = 21$ and $2n = 23$ polyhaploids combined more than two *Pennisetum* genomes. Therefore, triploid condition still may occur, more particularly for the locus or loci involved in apomixis expression. In the $2n = 28$ chromosome plants reported here that combine one genome from each maize and the wild species, diplosporous apomixis is normally expressed and functional in a diploid-like condition, suggesting that apomixis might be expressed in a diploid crop like maize.

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