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ORIGINAL ARTICLE

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Non-Mendelian transmission of an apospory-specific genomic region in a reciprocal cross between sexual pearl millet (*Pennisetum glaucum*) and an apomictic F1 (*P. glaucum* × *P. squamulatum*)

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Abstract We recently showed that aposporous apomixis, a form of gametophytic apomixis, is controlled by a single apospory-specific genomic region (ASGR) in both *Pennisetum squamulatum* and *Cenchrus ciliaris*. We present evidence that in a reciprocal cross between sexual pearl millet (*P. glaucum*) and an apomictic F1 (*P. glaucum* × *P. squamulatum*) the ASGR is not transmitted at the same rate. When pearl millet was used as the female parent and the apomictic genotype as the pollen donor, the ASGR was transmitted at a rate of 0.41 in a progeny of 57 plants, indicating a slight transmission ratio distortion. However, in a population of 52 rare sexual progenies characterized among a large progeny of a quasi-obligate apomict (an F1 hybrid of *P. glaucum* × *P. squamulatum*), the transmission rate of ASGR was only 0.12. This strong segregation distortion may have occurred at four different levels: (1) female meiosis, (2) during female gametophyte maturation, (3) upon fertilization with differential survival of embryos being a consequence of differential gene expression controlled by parent-of-origin specific effects (imprinting) and (4) at a later developmental stage of the embryo through an embryo/endosperm genetic incompatibility system.

Keywords Apomixis · Apospory · Segregation ratio distortion · Gamete lethality · Imprinting

Introduction

Apomixis is a genetically inherited trait by which plants are able to clone themselves through seeds without involvement of meiosis or fertilization of the egg (Asker and Jerling 1992). Apospory is the most common form of gametophytic apomixis in grasses (Brown and Emery 1958). Unreduced nucellar cells develop into ameiotic embryo sacs while the sexual pathway of megasporogenesis and development of reduced *Polygonum*-type embryo sacs abort (Nogler 1984). The unreduced embryo sacs in most aposporous grasses differentiate four nuclei with no antipodal cells, a feature easily identified through cytological observations. The unreduced egg cell develops parthenogenetically into an embryo. However, in some apomicts fertilization of the central cell remains a requirement in order to achieve formation of the endosperm and assure further development of the embryo (Nogler 1984), a process called pseudogamy.

We recently demonstrated that apospory is under the dominant control of an apospory-specific genomic region (ASGR) conserved in at least two grass species, *Pennisetum squamulatum* and *Cenchrus ciliaris* (Ozias-Akins et al. 1998; Roche et al. 1999). The ASGR is defined as a linkage group of several molecular markers for which virtually no recombination has been observed in either species. In a large progeny of 397 plants obtained from the cross between tetraploid pearl millet (*P. glaucum*) and hexaploid *P. squamulatum*, in which the latter was used as the pollen donor, we observed that the ASGR followed a tetrasomic inheritance with significant negative segregation distortion (Ozias-Akins et al. 1998). The nature of this distortion remains uncharacterized. The question arises whether the same phenomenon occurs during female meiosis. Insights on the transmission of ASGR in the female parent may shed light on some of the elements of apomixis, which seem to affect only female meiosis and subsequent development of embryo sacs.

Evaluation of the transmission of the ASGR through female meiosis, however, is complicated due to the dominant nature of this DNA region. Nearly complete penetrance of the apomictic trait limits the number of sexually-derived progenies to investigate. Only extensive crosses with a pollen donor carrying a dominant marker which facilitates early selection of scarce sexually-

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derived progenies has allowed such a study. We present here the results of large progeny tests conducted on an incompletely penetrant apomict and the molecular characterization (presence/absence of ASGR) of its sexually-derived progenies. Also we present data of the reciprocal cross in which the same apomictic genotype is used as pollen donor to establish a comparison of rates of transmission of ASGR between male and female.

Materials and methods

Plant material and cytological observations

An apomictic F1 progeny (290–124) was produced from the cross between an induced tetraploid pearl millet Tift 8677 (*2n*=4*x*=28) and *Pennisetum squamulatum* PS26 (2*n*=6*x*=54)(Ozias-Akins et al. 1998). All hybrids from this cross are expected to have 2*n*=41 chromosomes, 14 from pearl millet and 27 from *P. squamulatum* (Dujardin and Hanna 1983). F1 hybrid 290–124 has been propagated since 1995 either from vegetative clones or seeds. In cross A between pearl millet and 290–124, pollen collected from the latter was dusted onto the stigmas at anthesis of tetraploid pearl millet Tift 23. Self-pollinated progenies of pearl millet could be easily eliminated in early vegetative stages by leaf morphology (much broader width than in F1s and BC_1s).

In cross B, 2100 crosses were made in 1997 on 290–124 clones using pollen from the tetraploid pearl millet line with a dominant red marker trait (Tift 23; Hanna and Burton 1992). Dried frozen pollen harvested in the summer of 1995 was used as in Hanna (1994). Crossed inflorescences were threshed individually and progeny were tested for the presence of the red phenotype at the seedling stage. We found only 56 red-phenotype plants among approximately 25,000 progenies. Relative genome sizes of the 56 BC_1 individuals were determined by flow cytometry. A small piece of young, furled leaf was chopped with a razor blade in 1.0 ml of a detergent solution containing 0.1 M citric acid and 0.5% Tween 20 (Otto 1994). The chopped tissue was diluted with 3 ml of 0.4 M Na_2HPO_4 containing the DNA-specific fluorochrome DAPI (0.2 mg/100 ml) and filtered through a 40-µm sieve. Suspended nuclei were detected with a PAS-III flow cytometer (Partec, Münster, Germany) using the recommended setup for DAPI fluorescence. Each sample counted contained 7,000–11,000 fluorescent particles.

Inflorescences were collected at anthesis (stigma exsertion), fixed in FAA, and pistils were cleared with methyl salicylate (Young et al. 1979). The capacity to form aposporous embryo sacs was determined with differential interference contrast microscopy by observation of 20 ovules per genetic line. All ovules that contained multiple embryo sacs at anthesis, some of which clearly lacked antipodal cells, were scored as aposporous (Dujardin and Hanna 1984; Wen et al. 1998).

DNA extraction and PCR analysis

Leaf and shoot tissues from field- or greenhouse-grown plants were ground in liquid nitrogen and DNA was isolated according to Tai and Tanksley (1990) with some modifications (Ozias-Akins et al. 1993). All apospory-linked, PCR-based markers [sequence-characterized amplified regions (SCARs) A10H, O7M, P16R, Q8 M, R13, ugt197, U12H] used in this study have been described previously with respective annealing temperatures and thermocycling conditions (Ozias-Akins et al. 1998).

Analysis for restriction fragment length polymorphism

Plant DNA (15 µg) was digested overnight with 50 units of *Dra*I restriction enzyme (New England Biolab, Beverly, MA, USA) and separated by electrophoresis for 15 h at 3 V/cm in 1% GTG agarose (FMC, Rockland, ME, USA) in 1× TBE buffer. DNA was transferred to genescreen plus nylon membranes (NEN, Boston, MA, USA) by the alkaline transfer method (Sambrook et al. 1989). Hybridization was conducted with ^{32}P-labeled DNA generated by PCR amplification from plasmid DNAs using SCAR-specific primers. Hybridized blots were washed at a final stringency of 0.1× SSPE (0.18 M NaCl, 10 mM sodium phosphate pH 7.4, 1 mM EDTA) containing 0.1% SDS at 65 °C for 30 min.

Statistical analysis

Chi-square tests for the fixed-ratio hypothesis with two classes were conducted according to Gomez and Gomez (1984). The probability of a larger value of chi-square was determined using the John Pezzulbo JavaScript chi-square calculator accessed through http://davidmlane.com/hyperstat/chi_square.html.

Results and discussion

Cross A: *P. glaucum* × F1 line 290–124

From the cross between the tetraploid *P. glaucum* (female) and F1 interspecific hybrid 290–124 (male) we characterized 57 progenies by cytological, PCR and RFLP analyses (Figure 1). Among 57 progenies, 22 displayed the capacity to form aposporous embryo sacs as revealed by cytological examinations (Table 1). This included phenotypes with only aposporous embryo sacs as well as phenotypes with both polygonum-type and aposporous panicum-type embryo sacs. Upon observation of cleared ovules, these progenies with the potential to form aposporous embryo sacs differed in their internal morphology from those phenotypes that did not form aposporous embryo sacs (Fig. 2a–d). Approximately 60% of the ovules without aposporous embryo sacs in cross A and 30% in cross B displayed a slight to severe level of incomplete or aborted megasporogenesis or embryo sac development. Ovule abortion was a common phenomenon in sexual genotypes from a previous F1 cross be-

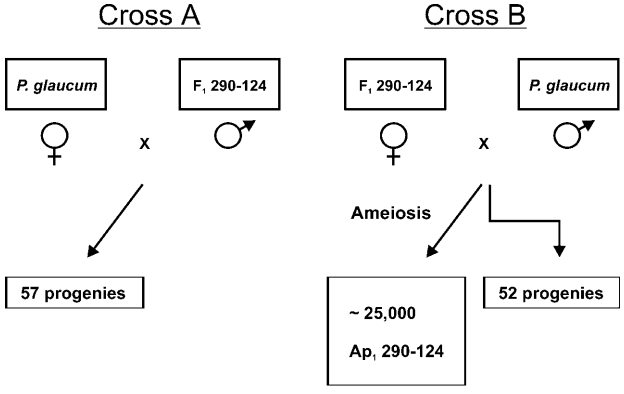


Fig. 1 Description of reciprocal crosses. **Cross A**: F1 290–124 (from *P. glaucum* × *P. squamulatum*) is used as pollen donor on pearl millet (*P. glaucum*). **Cross B**: Pearl millet is used as pollen donor on apomict F1 290–124. Approximately 25,000 progenies were clonal progenies of 290–124 (i.e, Ap_1 290–124) while only 52 *n*+*n* backcross progenies were identified

Table 1 Number of progenies from cross A [sexual *Pennisetum glaucum* × apomictic F1 (*P. glaucum* × *P. squamulatum*)] and cross B [apomictic F1 (*P. glaucum* × *P. squamulatum*) × sexual *P. glaucum*] classified cytologically into reproductive phenotypic classes and further analysed for presence of the ASGR-linked molecular markers, both SCARs and RFLPs

Cross A (sexual × apomict)			Cross B (apomict × sexual)		
Number of plants in each phenotypic class		Number of progenies with ASGR[a]	Number of plants in each phenotypic class		Number of progenies with ASGR[a]
With aposporous embryo sacs	22	22	With aposporous embryo sacs	4	4
Without aposporous embryo sacs	33	0	Without aposporous embryo sacs	46	0
Unclassified[a]	2	2	Unclassified[a]	2	2
Total	57	24	Total	52	6

[a] See text for definitions

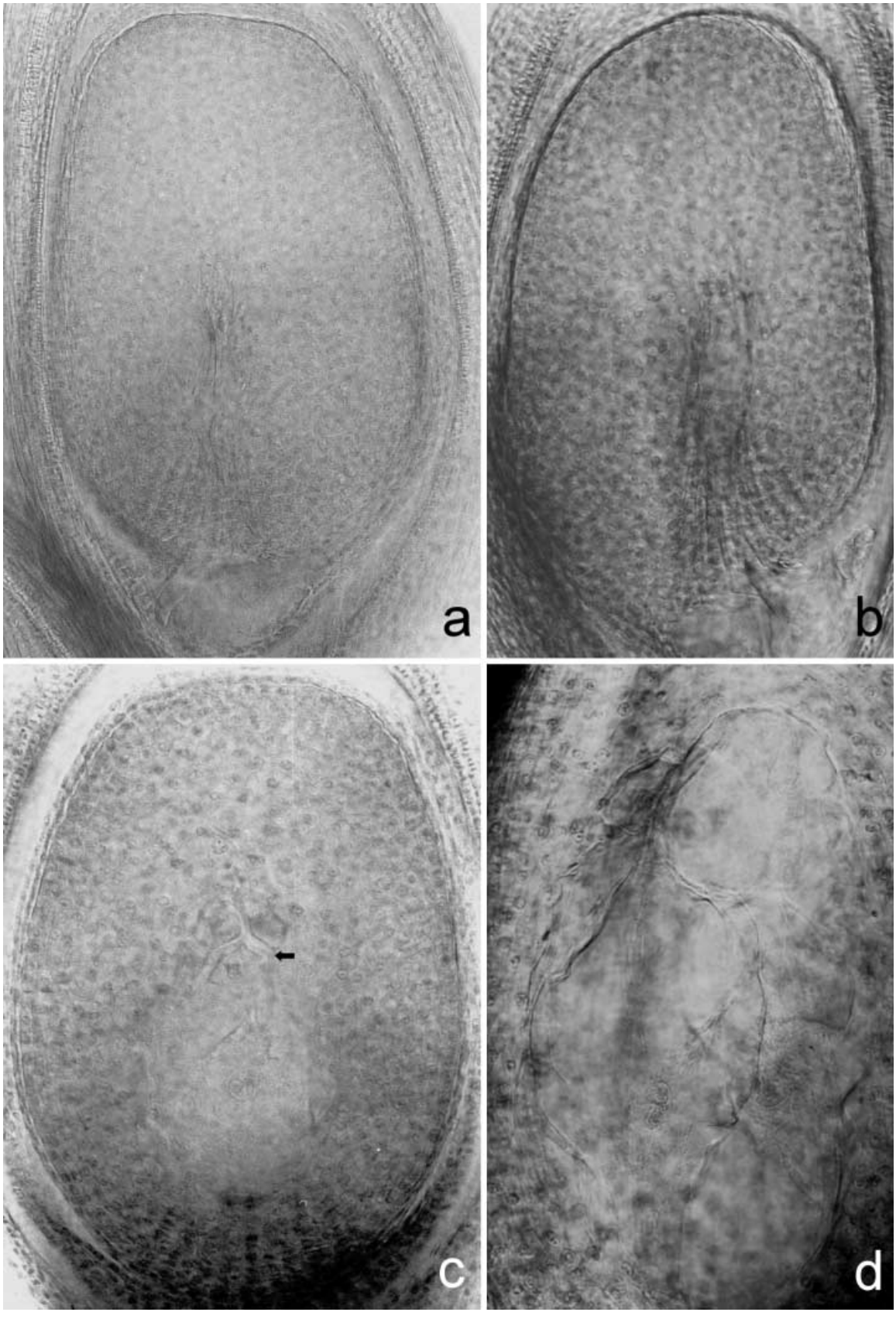
tween pearl millet and *P. squamulatum* (Dujardin and Hanna 1983). All progenies that formed aposporous embryo sacs had the ASGR as determined by PCR analyses with the seven ASGR-specific markers A10H, O7M, P16R, Q8M, R13, ugt197and U12H (Ozias-Akins et al. 1998). One example of PCR analyses conducted with the ugt197 marker is shown for 54 of the 57 progenies (Fig. 4A). No progeny without aposporous embryo sacs or having various degrees of ovule abortion harbored the ASGR (Table 1). Two progenies for which reproductive phenotype could not be determined cytologically due to the poorer quality of ovules tested were ASGR-positive, as revealed by multiple PCR analyses. As in two previous studies (Ozias-Akins et al. 1998; Roche et al. 1999), no recombination between ASGR and the apomictic mode of reproduction was found.

In cross A the observed rate of ASGR transmission was 0.42 (24/57). This was consistent with the ratio observed in a previous study with a similar cross, 4*x P. glaucum* × F1 hybrid 12Ap, in which 63/157 (40%) of BC_1 plants showed aposporous embryo sac development. (Dujardin and Hanna 1985). The 0.42 transmission rate for our BC_1 also was not significantly different (Chi^2=0.004, $P_{df=1}$=0.95) from a previously observed rate (0.41) in a population of 397 F1 individuals generated in a cross between *P. glaucum* and *P. squamulatum* (Ozias-Akins et al. 1998). With the latter cross involving paternal gametes from *P. squamulatum*, we found that the segregation ratio for ASGR best fit a model of tetrasomic inheritance with random chromatid segregation and lethality in duplex ASGR gametes. We could invoke and test at that time a tetrasomic inheritance model for ASGR in hexaploid *P. squamulatum*, since a previous study had shown the presence of primarily bivalent and quadrivalent pairing during male meiosis of this species (Dujardin and Hanna 1984) as well as the regular segregation of chromosomes to produce 27-chromosome gametes resulting in 41-chromosome F1 hybrids with tetraploid pearl millet (Dujardin and Hanna 1983). Chromosome segregation in the F1 during backcrossing was less regular, however, since only 50% of the BC_1s contained 35 chromosomes and the chromosome number of remaining progeny ranged from 32 to 39. Although no direct evidence is available for the pairing relationships of the linkage group bearing the ASGR, disomic inheritance of the ASGR might be modeled for the F1 290–124 based on chi-square analysis (1:1 expected ratio, χ^2= 1.42, $P_{df=1}$=0.233) and previous cytogenetic characterization of 41-chromosome F1 hybrids between pearl millet and *P. squamulatum* that documented the presence of primarily univalents and bivalents (Dujardin and Hanna 1983). However, it is also possible that transmission of the ASGR at a similar rate in two different genotypes (i.e., hexaploid *P. squamulatum* and F1 hybrid *P.glaucum* × *P.squamulatum*) is under the control of a meiotic drive mechanism. The ASGR could be made of supernumerary chromatin whose transmission remains constant (Beukeboom 1994).

Cross B: F1 line 290–124 × *P. glaucum* (red dominant marker)

From the multiple test-crosses between apomictic interspecific hybrid 290–124 and *P. glaucum* (tetraploid Tift23 homozygous for a red dominant marker) (Fig. 1) we retained 56 red-phenotype plants among approximately 25,000 progenies. Further analysis of these plants by flow cytometry (Fig. 3) allowed us to determine whether they originated from either fertilization of a reduced egg (*n*+*n*) or fertilization of an unreduced egg (2*n*+*n*)(BIII type; Rutishauser 1948). Only four plants were determined to have arisen from fertilization of an unreduced egg. The larger genome size of these plants (Fig. 3d) could be readily distinguished from that of the parental apomict 290–124 (Fig. 3a), a green maternal progeny plant from 290–124 (Fig. 3b), and the red BC_1s that arose from fertilization of a reduced egg (Fig. 3c). Fifty-two red plants were the products of fertilization of a rare reduced egg from 290–124 by a sperm cell from the millet genotype carrying the dominant marker. From characterization of these plants by cytological observations, PCR, and RFLP analysis (Table I), only six plants contained the ASGR as revealed by apomixis-linked molecular markers (Fig. 4B). The transmission ratio of the ASGR region was only 0.12 (6/52).

There was a highly significant deviation in the transmission of ASGR between the two populations of com-


a
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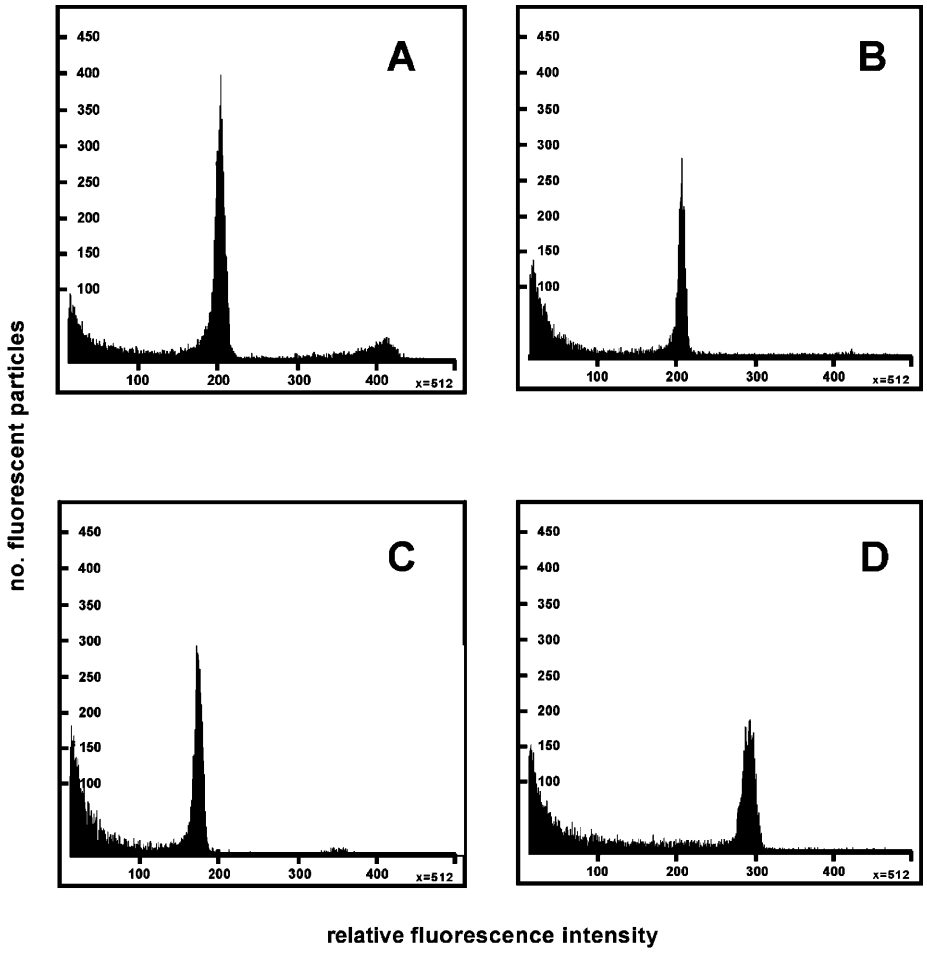

Fig. 3A–D Flow cytometric analysis of DAPI-stained nuclei from F1 290–124 (**A**), a green maternal progeny plant from 290–124 (**B**), a red BC_1 that arose from fertilization of a reduced egg (*n*+*n*) (**C**), and a red BIII type that arose from fertilization of an unreduced egg (2*n*+*n*) (**D**)

parable size generated in crosses A and B (χ^2 = 15.0, $P_{df=1}$<0.01). We observed a much higher transmission ratio distortion against the ASGR in sexual reproduction when the female parent was the apomict. In *Tripsacum*-maize hybrids, where the form of gametophytic apomixis is diplospory, there also is a report of such segregation distortion against the apomixis locus when the female parent is apomictic (Grimanelli et al. 1998). In the *Tripsacum*-maize introgression program, the apomict has always been used as the female parent since the F1 and its derivatives are male sterile.

We could hypothesize that transmission distortion of the ASGR may occur at four different phases of sexual reproduction and early embryo development. (1) At female meiosis: transmission distortion towards elimination of the ASGR at this stage may indicate that the ASGR suppresses meiosis. This negative influence may itself be an element of apomixis or due to an ASGR-linked lethal gene. This hypothesis would be consistent with the significant though small distortion against the ASGR observed in male meiosis of *P. squamulatum* (Ozias-Akins et al. 1998) and the F1 hybrid 290–124. For as yet uncharacterized reasons, the distortion in female meiosis would be exacerbated in the ovules. This observation of female-enhanced segregation distortion is contrary to that of Busso et al. (1995) where significant segregation distortion for chromosomal regions of pearl millet defined by molecular markers was observed only for male gametes of one parent in a reciprocal cross. (2) In megaspore and reduced embryo sac survival: these meiotically-reduced structures may not be as fit if they carry haploid nuclei with the ASGR. Obviously, this lack of fitness would contrast sharply with the apparent fitness of unreduced embryo sacs in the aposporous pathway of the same genotype. However, the ASGR may induce partial lethality when its effective dosage is increased by reduction of half of the genome content of a cell after meiosis. From our segregation studies we know that the ASGR already is in a simplex condition in *P. squamulatum* (Ozias-Akins et al. 1993,1998). In an attempt to explain the lower segregation ratio distortion observed in microspores, the ASGR in female gametophytes may be more deleterious to cells in close contact with surrounding unreduced maternal somatic tissues

◀ **Fig. 2a–d** Differential interference contrast microscopy of different reproductive phenotypes in progenies of crosses between pearl millet (*P. glaucum*) and F1 290–124 (*P. glaucum* × *P. squamulatum*). (**a**) Ovule with totally aborted meiosis; (**b**) Ovule with a partially aborted embryo sac; (**c**) Ovule with one *Polygonum*-type embryo sac. Note the antipodal cells (*arrow*). (**d**) Ovule with multiple *Panicum*-type embryo sacs

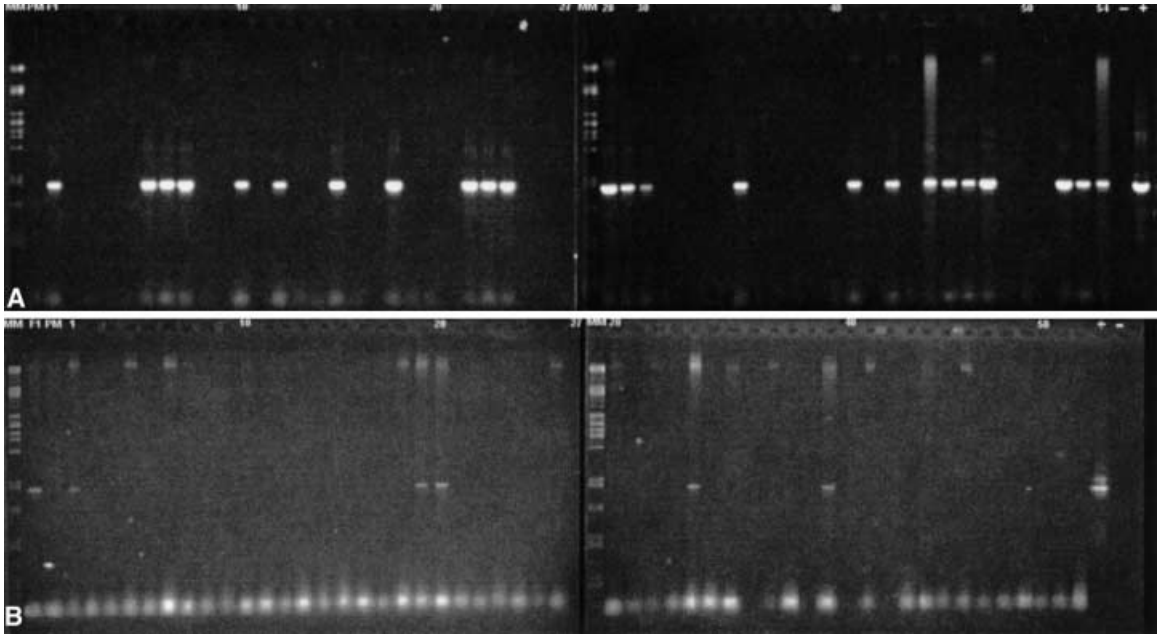

Fig. 4A, B Polymerase chain reaction analysis of progenies with SCAR P16R. **A.** Fifty-four progenies from cross A, *P. glaucum* × F1 290–124 (*P. glaucum* × *P. squamulatum*). **B.** Fifty-two progenies from cross B, F1 290–124 (*P. glaucum* × *P. squamulatum*) × *P. glaucum*. Control lanes on both: (*PM*) *P. glaucum;* **(**F_1**)** F1 290–124: *P. glaucum* × *P. squamulatum;* **(–)** negative control (no DNA); **(+)** positive control (SCAR P16 cloned in pGemT vector and used as template DNA). MM indicates a molecular weight DNA ladder from lambda DNA digested with *Pst*I. The amplified fragment is about 950 bp

than in the late development of free male gametophytes (McCormick 1993). On the contrary, the difference in frequency of apomictic BC_1 progeny of *Tripsacum* that arose from fertilized reduced eggs (0/218) versus parthenogenetically developing eggs (5/12; 41%)(Leblanc 1995; Grimanelli et al. 1998) suggests that meiotically-derived eggs containing an apomixis locus may not be selected against until after fertilization. (3) Upon fertilization through an imprinting phenomenon: the ASGR when transmitted through a reduced egg cell will induce more embryo lethality than when transmitted by a pollen grain. It has recently been shown that numerous paternally inherited alleles are not expressed in early embryo and endosperm development of *Arabidopsis thaliana*, while the corresponding maternally inherited alleles are expressed (Vielle-Calzada et al. 2000). If there are ASGR-linked genes that are detrimental to the zygote and early embryo stages, their expression in the maternal genome would be deleterious, but their silencing in the paternal genome would not reduce survival. (4) During early embryo development by some unknown embryo-endosperm interaction: the presence of one copy of the ASGR in the embryo and two copies of the ASGR in the endosperm may constitute an unfit combination. This situation occurs during sexual reproduction only when the ASGR is contributed by the female parent and not by the male parent. Similarly, fertilization of a binucleate central cell in an aposporous embryo sac, thus containing two copies of the ASGR, does not appear to lead to successful endosperm formation (Morgan et al. 1998).

Reconciling the observations in *Tripsacum* (Grimanelli et al. 1998) with ours is difficult. In this grass genus, the bias against apomictic progeny from fertilized eggs disappeared in the BC_3 generation, and it was hypothesized that in *Tripsacum* an incompletely penetrant transmission ratio distortion factor linked in repulsion with the apomixis locus had been eliminated. In *Pennisetum*, a single-dose lethality factor linked in repulsion should result in differences in female transmission of the ASGR among different F1 hybrids. This hypothesis would be difficult to test because of the large numbers of progeny from a near-obligate apomict that would have to be screened in order to find a sufficient number of sexually-derived progeny. Evidence against a lethality factor tightly linked in repulsion comes from the observation that offtype progeny from advanced backcross (BC_7) apomicts still are almost all sexual (W. Hanna, unpublished date).

Genetic typing by the polymerase chain reaction is a well-tested approach for human sperm cells (Leeflang et al. 1996). If this technique could be successfully applied to individual pollen grains, it would allow us to test whether segregation distortion through the male parent occurs during meiosis and gametophyte maturation or after fertilization. Testing female meiosis could perhaps be accomplished with in situ hybridization of the ASGR-specific DNA to sectioned ovules collected at the tetraspore stage. In an apomict with limited residual sexuality, large numbers of ovules would have to be examined in order to find the few which complete meiosis. Molecular characterization of the ASGR for gene content could provide tools to directly test the differential expression of potentially imprinted genes.

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