Delineation by Fluorescence *in Situ* Hybridization of a Single Hemizygous Chromosomal Region Associated With Aposporous Embryo Sac Formation in *Pennisetum squamulatum* and *Cenchrus ciliaris*

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ABSTRACT

Apomixis is a means of asexual reproduction by which plants produce embryos without meiosis and fertilization; thus the embryo is of clonal, maternal origin. We previously reported molecular markers showing no recombination with the trait for aposporous embryo sac development in *Pennisetum squamulatum* and *Cenchrus ciliaris*, and the collective single-dose alleles defined an apospory-specific genomic region (ASGR). Fluorescence *in situ* hybridization (FISH) was used to confirm that the ASGR is a hemizygous genomic region and to determine its chromosomal position with respect to rDNA loci and centromere repeats. We also documented chromosome transmission from *P. squamulatum* in several backcrosses (BCs) with *P. glaucum* using genomic *in situ* hybridization (GISH). One to three complete *P. squamulatum* chromosomes were detected in BC_6, but only one of the three hybridized with the ASGR-linked markers. In *P. squamulatum* and in all BCs examined, the apospory-linked markers were located in the distal region of the short arm of a single chromosome. All alien chromosomes behaved as univalents during meiosis and segregated randomly in BC_3 and later BC generations, but presence of the ASGR-carrier chromosome alone was sufficient to confer apospory. FISH results support our hypotheses that hemizygosity, proximity to centromeric sequences, and chromosome structure may all play a role in low recombination in the ASGR.

APOMIXIS in flowering plants is an asexual mode of reproduction that results in the formation of seeds containing an embryo with the maternal genotype (Asker and Jerling 1992). Apomixis has both sporophytic (adventitious embryony) and gametophytic forms (Koltunow 1993). In adventitious embryony a new sporophyte arises from a somatic cell of the ovule. Gametophytic apomixis can result from diplospory, in which the unreduced embryo sac originates from a generative cell, or apospory, in which the embryo sac develops from somatic cells of the ovule (Grimanelli *et al.* 2001). After embryo sac development, parthenogenesis occurs in both forms of gametophytic apomixis. Apomixis has been described in at least 33 of the 460 families of angiosperms (Carman 1997), but has been most frequently observed in the Poaceae, Asteraceae, and Rosaceae (Richards 1986).

Apomixis has vast potential for application to breeding and propagation of crop plants, although the potential is far from realized. This reproductive strategy enables the production of clonal seed from a particular genotype, even one that is highly heterozygous, thus immortalizing hybrid vigor. Unfortunately, no cultivated crop displays a sufficient degree of apomixis for practical application, and only a few crop plants have wild relatives that are near-obligate apomicts. Considerable efforts have been made to introduce the trait into crops such as maize (Leblanc *et al.* 1995; Savidan 2001) and pearl millet (Dujardin and Hanna 1989) by traditional breeding involving interspecific hybridization. The most successful of these hybridization programs has used *Pennisetum squamulatum* Fresen, an apomictic relative of the domesticated plant, pearl millet [*P. glaucum* (L.) R. Br.]. Pearl millet is grown for its grain primarily in Africa and India and as a forage crop in tropical and subtropical regions of the world, including the southern United States. The transfer of apomixis from *P. squamulatum* to *P. glaucum* has been pursued for the last two decades, and the crossing scheme that led to the recovery of a backcross 3 (BC_3) individual showing near-obligate apomixis has been described (Dujardin and Hanna 1989). Subsequently, additional backcross generations (BC_4–BC_7) were produced. The transfer of apomixis from *P. squamulatum* to pearl millet has been hindered by linkage drag of undesired characteristics, among them low seed set (~5% for open-pollinated BC_3 and later generations) and high (~60%) pollen

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sterility. Genetic and cytogenetic analyses of these apomictic backcross lines have been carried out to study their chromosome behavior and the inheritance of apomixis (Dujardin and Hanna 1989; Hanna *et al.* 1993). All of these backcross lines have 27–29 chromosomes, but traditional cytogenetic investigation has not been able to distinguish the alien chromosomes from the pearl millet chromosomes.

An alternative approach to producing apomictic crops might be to transfer to a sexual plant one or more well- characterized genes known to confer the trait of apomixis. In the search for such genes, information about the molecular and genetic basis of apomixis in nondomesticated species is being accumulated. Genetic and molecular mapping studies based on the analysis of offspring from apomictic by sexual crosses have been carried out on multiple species reproducing by gametophytic apomixis (Grossniklaus *et al.* 2001). Aposporous apomixis in *Panicum maximum* (Savidan 1982), *Ranunculus auricomus* (Nogler 1984), *Cenchrus ciliaris* (Sherwood *et al.* 1994), *Brachiaria decumbens* (Do Valle and Miles 2001), *Paspalum simplex* (Caceres *et al.* 2001), and *P. squamulatum* (Ozias-Akins *et al.* 1998) is inherited as a single, dominant Mendelian factor. Inheritance of diplosporous apomixis in Taraxacum and Erigeron has been shown to be a multilocus phenomenon where the development of diplosporous embryo sacs and parthenogenesis can be genetically separated (van Dijk *et al.* 1999; Noyes and Rieseberg 2000).

Our lab has centered its research on two grasses, *P. squamulatum* Fresen and *C. ciliaris* L. [syn. *P. ciliare* (L.) Link; buffelgrass], both of which reproduce by apospory. The phylogeny of the "bristle-grass" clade of panicoid grasses has been investigated recently (Duvall *et al.* 2001; Giussani *et al.* 2001; Doust and Kellogg 2002). Current evidence supports the idea that Cenchrus and Pennisetum are paraphyletic genera although both fall in a larger monophyletic clade.

As part of a mapping study, we isolated 12 sequence-characterized amplified region (SCAR) markers that showed no recombination with an apospory-specific genomic region (ASGR) in *P. squamulatum* (Ozias-Akins *et al.* 1998). Ten of the SCARs were conserved and nine showed no recombination with the ASGR in *C. ciliaris* (Roche *et al.* 1999). Several of the SCAR markers were shown by Southern hybridization to be hemizygous with no apparent allele on chromosomal homologs transmitted to the sexual offspring. Bacterial artificial chromosome (BAC) libraries were constructed from the two apomictic lines under study and used for preliminary physical mapping. Classes of BAC clones grouped by apomixis-linked SCAR markers did not overlap, which predicts that building a contig spanning the apomixis locus likely will require multiple walking steps (Roche *et al.* 2002). Given the lack of recombination within the ASGR, its apparent large size, and the lack of a high-density genetic map for either species, it became important to visualize the physical location of the ASGR on chromosomal spreads using fluorescence *in situ* hybridization (FISH) and to trace its transmission to offspring from several backcross generations.

In this article, we present data on the hybridization of metaphase chromosomes with ASGR-linked BAC clones and with pooled ASGR-linked markers to determine the physical location of the ASGR in two apomictic species, *P. squamulatum* and *C. ciliaris*, as well as apomictic backcross derivatives from crosses of *P. glaucum* by *P. squamulatum*. Furthermore, we compare the location of the ASGR with respect to rDNA loci and centromeric repeats in three genotypes. Finally, we examine the transmission and frequency of the ASGR-carrier chromosome in apomictic backcrosses. FISH results have proved to be instructive in guiding our interpretation of the basis for low recombination, have allowed new insights into the evolution of chromosomal structure in two apomictic grasses, and have determined that a single chromosome is sufficient for the transmission of apomixis and molecular markers linked to the trait.

MATERIALS AND METHODS

Genetic stocks: Pennisetum species used in this study included *P. squamulatum* (PS26; PI 319196; $2n = 56$) and an induced tetraploid pearl millet (*P. glaucum*, $2n = 4x = 28$). Progeny from apomictic and sexual backcross lines used in this study were grown in the field in the summers of 1999 and 2000 (for collecting inflorescences containing various stages of meiosis) and in the greenhouse in the winter of 2000 (for collecting root tips). *C. ciliaris*, B12-9, is an obligate apomict derived from open pollination of a sexual buffelgrass plant, B2-S (Sherwood *et al.* 1994).

Chromosome preparation: *Mitotic chromosome spreads:* Root tips were collected and incubated in tap water saturated with α-bromonaphthalene for 2–4 hr and subsequently fixed in fresh ethanol:acetic acid (3:1) for a minimum of 2 days before use. Fixed root tips were briefly rinsed in 30 mm citrate buffer, pH 4.5, the root caps were removed, and the meristimatic region was incubated in 0.3% cellulase RS (Karlan Research, Torrance, CA), 0.3% pectolyase Y23 (Karlan Research), and 0.3% cytohelicase (Sigma-Aldrich, St. Louis) in 30 mm citrate buffer, pH 4.5 (Zhong *et al.* 1996), at 37° for 1.5–3 hr. After a short rinse in the same buffer, the root tips were squashed in 60% acetic acid under a coverslip. The slides were then frozen in liquid nitrogen and the coverslip was removed with a razor blade. The slides were further incubated in 60% acetic acid for 15 min at room temperature, dehydrated in ethanol, and air dried. The slides were stored at −20° until used.

Meiotic chromosome spreads: Inflorescences protruding about one-third of their length from the boot were collected from *P. squamulatum*, *P. glaucum*, BC_3, BC_4, BC_5, BC_6, and BC_7. After checking the stage of meiosis in each inflorescence by squashing anthers in acetocarmine, inflorescence sections containing meiotic cells at metaphase I were fixed in ethanol:acetic acid (3:1) and stored at 4°. Up to 1–2 months after fixation, florets were removed from the fixative and soaked in 30 mm citrate buffer (pH 4.5) for 5–10 min. Dissected anthers were cut at the apex and squeezed with a surgical knife to extrude the pollen mother cells (PMCs) into a 10 × 35-mm petri dish containing 1 ml of 30 mm citrate buffer (pH 4.5). PMCs were pipetted into a 1.5-ml microcentrifuge tube where digestion was carried out in 50 μl of enzyme mixture (as above) at 37° for 30–45 min. Digestion time was dependent on the length

of time materials had been stored in fixative. Digested PMCs were collected by centrifugation at 600 × *g* for 5 min at room temperature. The supernatant was removed, and PMCs were resuspended in a volume of 60% acetic acid equal to three times the volume of the digestion solution and incubated for 10 min on ice. PMCs were again collected by centrifugation and resuspended in 4 μl of 60% acetic acid for each slide. Usually one slide was made from each anther by applying the 4 μl of PMC suspension to a precleaned slide and covering it immediately with a 22 × 22-mm cover glass. The cover glass was removed after freezing, and the spread was dehydrated in an ethanol series and air dried.

DNA probes: *Genomic in situ hybridization and pooled marker probes:* Genomic DNAs were isolated using the method described in Ozias-Akins *et al.* (1993) and purified with phenol:chloroform:isoamyl alcohol (25:24:1) extraction. Plasmid DNAs containing 12 molecular markers mapped to the ASGR (Ozias-Akins *et al.* 1998) were isolated by alkaline lysis minipreps (Sambrook *et al.* 1989). Equal amounts (by weight) of DNA from each plasmid were mixed together and 1.5 μg of the mixture was labeled as an ASGR-specific probe.

ASGR-linked probes: The construction of the "polyhaploid" and "buffelgrass" BAC libraries, containing the ASGR from *P. squamulatum* and *C. ciliaris*, respectively, has been reported (Roche *et al.* 2002). The polyhaploid library was derived from the polyhaploid apomictic line MS228-20 that was germinated from seeds of an open-pollinated apomictic polyhaploid F_1 line derived from a cross between *P. glaucum* and *P. squamulatum* (Dujardin and Hanna 1986). The buffelgrass library was derived from the apomictic line B12-9 (Sherwood *et al.* 1994). ASGR-linked BAC clones from both libraries were identified through screening the BAC library filters with ^{32}P-labeled ASGR-linked SCAR probes. Linkage of individual BACs to the ASGR was confirmed through the analysis of ASGR-specific SCAR or restriction fragment length polymorphism markers (Roche *et al.* 2002). To select ASGR-linked BACs containing low-copy sequences for FISH, Southern blots containing ~1 μg of *Hin*dIII-digested DNA from each BAC clone that had been fractionated on an agarose gel were probed with ^{32}P-labeled total genomic DNA from BC_3 for the polyhaploid clones or B12-9 for the buffelgrass clones (Zwick *et al.* 1997). BAC clones that showed little or no signal were used for plasmid preps (QIAGEN, Valencia, CA).

Centromeric probes: Two highly repetitive *Kpn*I fragments of ~140 and 160 bp have been reported in the genus Pennisetum (Ingham *et al.* 1993). The two fragments differ primarily by an 18-bp deletion in the 140-bp *Kpn*I family. A 137-bp *Hae*III repeat from *P. glaucum* cv. Massue has been previously shown to localize to the centromere (Kamm *et al.* 1994). The 160-bp *Kpn*I fragment contains ~93% sequence similarity to the 137-bp *Hae*III repeat and therefore was expected to hybridize with centromeric sequences. The 160-bp *Kpn*I fragment was used as a centromeric probe for FISH analysis in BC_3 and *P. squamulatum*. For *C. ciliaris*, a centromeric probe was obtained by screening the BAC library with a 160-bp *Kpn*I fragment. DNAs from six to eight of the BAC clones that showed the strongest hybridization signals were digested with *Kpn*I and *Hae*III. The centromeric sequence content was further confirmed by hybridizing the *Kpn*I- and *Hae*III-digested BAC DNAs with a ^{32}P-labeled 160-bp *Kpn*I clone. The BAC clone containing the largest proportion of laddered fragments was chosen as a centromeric probe for FISH analysis (data not shown).

Ribosomal DNA probe: Plasmid pTA71 containing a 9.5-kb *Eco*RI 18S-5.8S-25S repeat unit from wheat was used to detect rDNA (Gerlach and Bedbrook 1979).

Probe labeling, slide pretreatment, and hybridization: DNAs were labeled with either biotin (bio)-11-dUTP (Roche, Indianapolis) or digoxigenin (dig)-11-dUTP (Roche), using a nick translation kit (Roche). Labeled probes were purified by two sequential ethanol precipitations in the presence of 2.1 M ammonium acetate. Pelleted DNA was resuspended in 100% formamide for storage at −20°.

On the day of hybridization, slides were dried at 60° for 30 min. Subsequent pretreatment steps to partially digest RNA and proteins were as described by Zhong *et al.* (1996). Probes were mixed in pairwise combinations for double target hybridization experiments. The hybridization mix for each slide consisted of 1.5–7 ng/μl of probe for each target, 50% formamide, 10% dextran sulfate, 100–300 ng/μl salmon sperm DNA and 2× SSC in a final volume of 10–15 μl. For genomic *in situ* hybridization (GISH), 100 ng/μl sheared *P. glaucum* DNA was used as blocking DNA. The hybridization stringency was reduced for the 160-bp *Kpn*I fragment by adjusting the formamide and SSC concentrations to 25% and 5×, respectively. The hybridization mixtures were denatured at 100° for 10 min and snap chilled on ice. The chromosome preparations with the hybridization mix were covered with a coverslip and denatured at 80° for 2 min. Slides were incubated at 60° in a moist chamber for 1 hr and then allowed to cool down to 37° (for FISH) and 39°–42° (for GISH) where they subsequently were incubated for 16–20 hr. Two posthybridization washes were performed in 50% formamide in 2× SSC at 42° for 15 min, except for the 160-bp *Kpn*I fragment where posthybridization washes consisted of 20% formamide in 5× SSC. Formamide washes were followed by room temperature washes in 2× SSC prior to detection.

Probe detection: Two-color detection was carried out according to Zhong *et al.* (1996) with modifications. The dig-labeled probes were detected with FITC by using a fluorescent enhancement kit (Roche) while bio-labeled probes were detected using avidin Texas red (Vector Laboratories, Burlingame, CA). All slides were blocked with 5% nonfat dry milk in 1× PBS (buffer 4M) at 37° for 30 min. All further incubation steps were performed at 37° for 30 min, each followed by three washing steps of 5 min each with T-PBS [0.2% Tween 20 (v/v) in 1× PBS; Fisher Scientific, Pittsburgh] at room temperature. For BAC-FISH, incubation steps were carried out in the following order: Texas red-conjugated avidin; anti-dig IgG from mouse; anti-mouse IgG from sheep conjugated with dig and anti-avidin IgG from goat conjugated with biotin; sheep anti-dig, fluorescein-conjugated fab fragment, and Texas red-conjugated avidin. Since signal amplification was not required for GISH, only the last step was carried out. The slides were given a final rinse in 1× PBS and mounted in Vectashield (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (DAPI; 1.5 ng/μl).

Slides were examined under an Olympus BX50 fluorescence microscope. A minimum of 20 spreads for BAC-FISH and 5 spreads for GISH were examined for each slide. More than 80% of the spreads produced discrete signals. Fluorescent signals were detected for DAPI (λ_{ex} = 360 nm, λ_{em} = 420 nm), FITC (λ_{ex} = 480 nm, λ_{em} = 515 nm), and Texas red (λ_{ex} = 560 nm, λ_{em} = 645 nm), and monochrome digital images were captured with a charge-coupled device camera (SenSys, Photometrics, Tucson, AZ). Images were pseudocolored with blue for DAPI, green for FITC, and red for Texas red. Images were compiled with Image Pro Plus, version 4 for Win 95/98 (Media Cybernetics, Silver Spring, MD). Final adjustments were made using Adobe Photoshop version 5.0. Chromosome lengths were obtained with the image analysis programs, Object-Image2.08 and CHIAS III (Kato and Fukui 1998).

RESULTS

Generation of stocks segregating for apomixis: The trait for apomixis was introduced from *P. squamulatum* into the sexual species *P. glaucum* according to the recur-

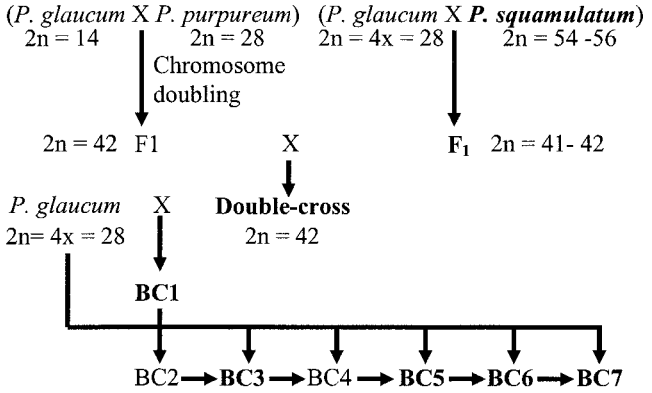

FIGURE 1.—Pedigree summary of hybrids and backcrosses used for FISH/GISH. Materials used for this investigation are shown in boldface type. The F_1 of *P. glaucum* × *P. purpureum*, BC_2, and BC_4 were not included in these experiments. Chromosome doubling was accomplished with colchicine treatment.

rent backcrossing scheme diagrammed in Figure 1. Backcross generations 1–7 were generated and screened according to the methods outlined in DUJARDIN and HANNA (1989). Since a double cross hybrid of (*P. glaucum* × *P. purpureum*) × (*P. glaucum* × *P. squamulatum*) was used early in the crossing scheme to increase male fertility in the backcrosses (DUJARDIN and HANNA 1984a), the apomictic double cross also was included in this study. Mode of reproduction could be determined by examining ovules cleared in methyl salicylate for aposporous embyo sac development (YOUNG *et al.* 1979) and by scoring the uniformity of progeny in the field or greenhouse. Since these were heterozygous plants, variable progeny indicated some degree of sexuality.

Comparison of *P. squamulatum* and *P. glaucum* chromosomes in interspecific hybrids: *P. squamulatum* apparently shares similar DNA sequence with *P. glaucum* at telomeric and centromeric regions since these regions on the chromosomes of *P. squamulatum* were blocked with *P. glaucum* genomic DNA (Figure 2, A and B). We observed 56 chromosomes in *P. squamulatum*, accession PS26 (Figure 2A), contrary to the previous reports of 54 chromosomes in most other accessions ($2n = 6x = 54$; RAMAN *et al.* 1959; PATIL *et al.* 1961; SISODIA 1970; RANGASWAMY 1972; DUJARDIN and HANNA 1984b). SINDHE (1976) observed two supernumerary chromosomes in this species that were rod-like and acrocentric or submetacentric and that were largely eliminated during meiotic cycles. Of the 56 chromosomes that we observed in *P. squamulatum* accession PS26, none fit the description of the supernumerary chromosomes of SINDHE (1976). GISH with biotinylated genomic DNA of *P. squamulatum* confirmed that chromosomes from *P. squamulatum* were introduced into *P. glaucum* through crossing of the two distantly related species. Although we examined the chromosome number in only four F_1 individuals, all showed 28 *P. squamulatum* chromosomes (differentiated by GISH), which suggests that regular disjunction occurred during meiosis and that subsequent transmission to progeny was normal. The origin of the two additional chromosomes in PS26 remains undetermined, but their presence or absence has no apparent effect on apomixis. A total of 42 chromosomes, including 28 *P. squamulatum* chromosomes and 14 *P. glaucum* chromosomes, were observed in both apomictic and sexual F_1 individuals (Figure 2, B and C, respectively). We used F_1 plants to investigate the chromosome characteristics of *P. squamulatum* since each F_1 contained half of the chromosomes of *P. squamulatum*, and chromosomes from both species could be directly compared in F_1 hybrids. The size range in *P. squamulatum* and *P. glaucum* was 9.87–3.95 μm and 10.46–5.87 μm, respectively. The length changes in *P. squamulatum* formed almost a continuous gradient from the largest to the smallest chromosome. Most of the chromosomes were metacentric or submetacentric (Figure 2, B and C). It was not possible to unambiguously identify the potential homo(eo)logous chromosomes on the basis of morphology.

Pearl millet (*P. glaucum*) is one of the two species in section Penicillaria with a base chromosome number of $x = 7$ (STAPF and HUBBARD 1934; JAUHAR 1981). There were seven pairs of chromosomes from *P. glaucum* in the F_1 hybrid with *P. squamulatum*. Only three chromosome pairs could be distinguished easily from others using morphological features [1, longest and metacentric; 2, submetacentric; and 7, shortest and subtelocentric with a satellite on the short arm (Figure 2, E and K)]. For the *P. glaucum* chromosomes, the percentage of the total haploid chromosome length in the F_1 ranged from 9.8% (shortest chromosome) to 17.8% (longest chromosome), which is consistent with a previously published karyotype from KHALFALLAH *et al.* (1993).

Transfer of *P. squamulatum* chromosomes into *P. glaucum* through backcrossing: Chromosome preparations were made from seven different generations (including *P. squamulatum*, F_1, BC_1, BC_3, BC_5, BC_6, and BC_7), 20 different lines, and 2–22 plants from each. Both metaphase I of meiosis and mitotic metaphase plates were investigated with the combination of FISH and/or GISH (Table 1). The number of *P. squamulatum* chromosomes was progressively reduced as backcrossing advanced with the recurrent parent, *P. glaucum*. In BC_1, differential labeling of genomic DNAs from *P. squamulatum* and *P. purpureum* (a bridging species used as a parent prior to the backcrossing step in the introgression program) resulted in hybridization of both probes to the same 14 chromosomes that could be observed by the yellow signal from the combined red and green fluorescence (Figure 2D). This result indicates that there is strong sequence similarity among repeats in the two genomes. Also in BC_1, the number of chromosomes that were blocked with pearl millet DNA increased to 22 (Figure

2D). At the BC_3 generation, three chromosomes and one segment of a fourth chromosome hybridized with *P. squamulatum* genomic DNA (Figure 2, E and L).

Alien chromosome transmission in recurrent backcrosses was determined with a combination of FISH and karyotyping. The characteristics of chromosomes in BC_3 that hybridized with *P. squamulatum* genomic DNA (alien chromosomes) or were blocked by *P. glaucum* DNA are shown in Figure 3 and Table 2. Although it is difficult to distinguish between the chromosomes of pearl millet and the alien chromosomes by only the length data (Table 2), the condensation levels revealed upon DAPI staining are substantially different (Figure 3). The ASGR-carrier chromosome has two moderately condensed regions, one around the centromere and another on the short arm. It is possible to recognize this chromosome in BC_3 solely on the basis of its morphology. Genomic DNA of *P. purpureum* also hybridized to the three to four alien chromosomes of BC_3 when it was used as one of the probes (Figure 2K). Beyond the BC_3 generation, plants classified as apomictic (*i.e.*, producing aposporous embryo sacs, including both obligate and facultative outcomes) contained one to three chromosomes that hybridized with *P. squamulatum* DNA, and the number of *P. glaucum* chromosomes ranged from 26 to 28 (Figure 2, F–J). In these materials, the total chromosome number was 28 or 29.

GISH of meiotic metaphase I indicated that the alien chromosomes in BC_3 and later backcross generations did not pair and thus are presumed not to be homologous to each other. They also were not homologous to any chromosome of *P. glaucum* because they were exclusively observed as univalents that lagged at anaphase I (Figure 2, L and M) or assorted to either pole randomly (Figure 2, N and O).

Selection of ASGR-linked BAC clones for FISH: Twenty-six polyhaploid and 17 buffelgrass ASGR-linked BAC clones that were isolated from the polyhaploid and buffelgrass BAC libraries (Roche *et al.* 2002) were tested for their repetitive DNA content by hybridization with total genomic DNA from the respective parental species. Nineteen of the 26 ASGR-linked polyhaploid BAC clones contained a low amount of repetitive DNA. Among the 17 buffelgrass ASGR-linked BAC clones, 12 were low in repetitive DNA content. The BACs used for this FISH study have been summarized in Table 3. The BACs were designated by "p" or "c" if they were derived from the polyhaploid or *C. ciliaris*, respectively. Fingerprinting analysis showed no overlap in either species between BAC clones containing different SCAR markers (Roche *et al.* 2002). In addition, BAC clones carrying the same SCAR designation can be from duplicated regions within the ASGR (Roche *et al.* 2002). Each BAC clone for which results are presented produced a discrete signal with a good signal/noise (s/n) ratio without the use of C_0t DNA. Cytoplasm was the major factor affecting the quality of the signal produced. Other factors that affected the s/n ratio included nonspecific binding of antibody and the probe quality.

A single chromosome transmits the ASGR to backcross progeny: Twelve ASGR-linked molecular markers were pooled, labeled with digoxigenin, and probed simultaneously with labeled *P. squamulatum* DNA onto the materials as shown in Table 1. Six of the markers were known to be low-copy-number DNAs and the others were repetitive sequences (Ozias-Akins *et al.* 1998), but the distribution of the repeats across the genome had not been determined. The FISH signal from these pooled markers was consistently observed at the end of a single, metacentric *P. squamulatum* chromosome (long arm to short arm ratio of 1.34 ± 0.19; Figure 2, A, B, and E–H, green, arrowheads). Plants with the terminal ASGR-specific signal were later classified as either obligate or facultative apomicts. Plants with only meiotically derived embryo sacs did not show the terminal ASGR signal on any chromosome, although other alien chromosomes were sometimes present as shown by GISH. It is likely that the signal from the ASGR-linked markers was derived mainly from the repetitive members of the pool since a probe pool that eliminated the single copy markers gave the same result (data not shown).

Two-color FISH was used to map the comparative positions of ASGR-linked BAC clones containing different SCAR markers. Chromosome spreads from BC_3 were most extensively examined with BAC-FISH. In BC_3, all the ASGR-linked polyhaploid BACs listed in Table 3 localized to the distal end of a single chromosome. FISH results from a subset of the ASGR-linked BAC clones are shown in Figure 4, A–C. In each case of dual target mapping, an overlapping (yellow) signal was detected when the FITC (green) and Texas red (red) signals from BAC clones containing different SCAR markers were merged. On highly condensed root-tip metaphase chromosomes of BC_3, the single hybridizing chromosome showed no separation in signal sites between any of the ASGR-linked BAC clones (Figure 4A). Although not directly tested, it is unlikely that the single chromosome that showed signal was derived from pearl millet and not *P. squamulatum* as the pearl millet background is autotetraploid and therefore should have yielded typically four chromosomes with signal. Because the ASGR in BC_3 was introgressed from *P. squamulatum*, PS26 ($2n = 56$), it also was important to test the number and location of hybridization sites in this apomictic parent and to compare the results with BC_3.

To determine if introgression of the ASGR in BC_3 involved any structural rearrangement of the hybridizing *P. squamulatum* chromosome, FISH with ASGR-linked BAC clones also was performed on *P. squamulatum*. Only a subset of the ASGR-linked BAC clones [p109 (Q8M) and p207 (ugt197)] were hybridized and detected in pairs using two-color FISH as described above for BC_3. Both BAC clones again hybridized to the distal end of a single chromosome with no detectable signal

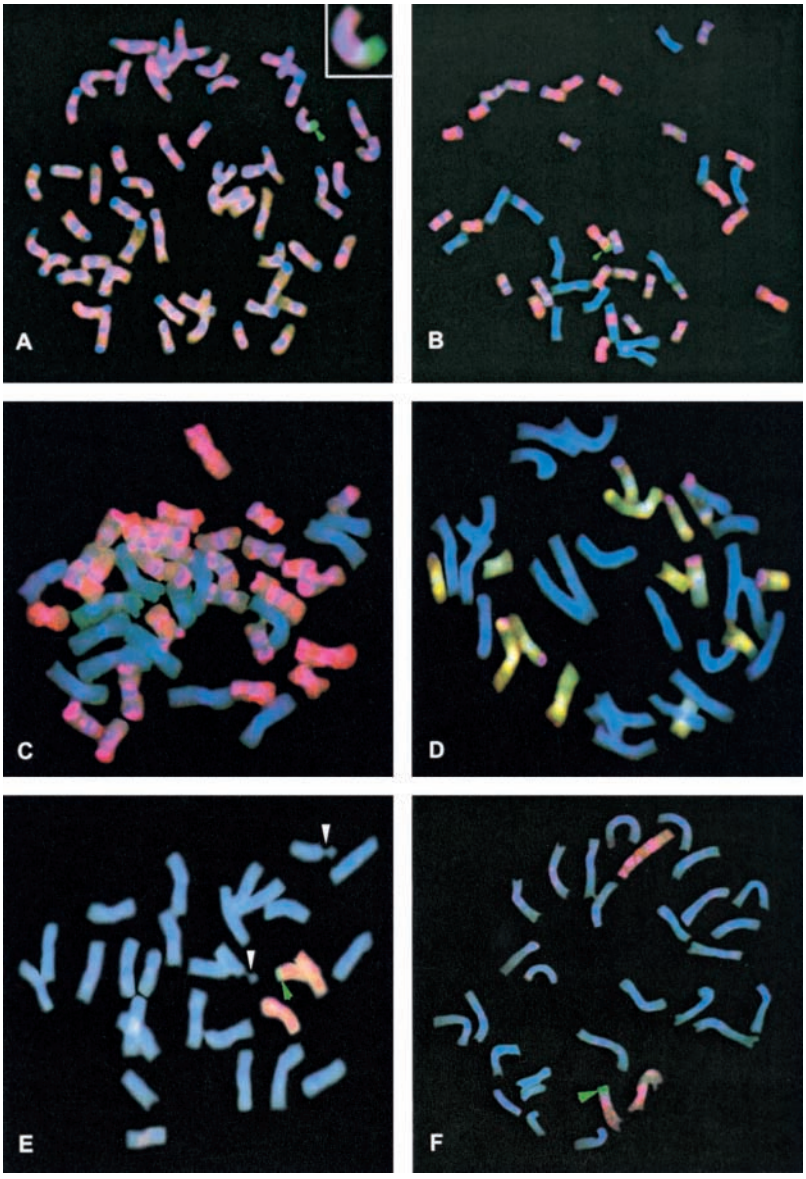

FIGURE 2.—Results of GISH and FISH on chromosomes from materials in the pedigree shown in Figure 1. White arrowheads indicate chromosomes with satellites. Shown is hybridization of mitotic (A–K) and meiotic (L–O) chromosome preparations with dual-labeled probes of *P. squamulatum* genomic DNA (red) and the mixture of 12 ASGR-linked markers (green; A–C and E–H); *P. squamulatum* genomic DNA (red) and *P. purpureum* genomic DNA (green; D and K); or *P. squamulatum* genomic DNA alone (green; I, J, and L–O). Because both *P. squamulatum* and *P. purpureum* genomic DNAs hybridized to the same chromosomes, the color of the alien chromosomes in D and K appears yellow. Blue chromosomes were blocked with genomic DNA of *P. glaucum*. (A) *P. squamulatum*; (B) apomictic F_1 290-181D; (C) sexual F_1 290-105; (D) BC_1; (E) BC_3; (F) BC_6 43-1; (G) BC_5 44-4; (H) BC_6 62-1; (I) BC_7 60-9; (J) BC_5 44-1; (K) BC_3; (L) BC_3 J34; (M) BC_7 J57-3; (N) BC_6 J35; (O) BC_7 J57-2.

on other chromosomes (Figure 4B). Similar to BC_3, the two BAC signals overlapped. No detectable structural rearrangement was observed when the hybridizing chromosome was recorded either in its native *P. squamulatum* genetic background or in that of its introgression host.

In *C. ciliaris*, apomict B12-9 ($2n = 36$), the ASGR-linked BAC clones containing Q8M [c001] and ugt197 [c101], produced an overlapping signal on a single chromosome (Figure 4C). Unlike *P. squamulatum*, the position of the signal on the chromosome arm was not distally located.

Location of the ASGR relative to the centromere: Many Pennisetum sp. chromosomes are small and highly condensed at mitotic metaphase. Under such circumstances the centromere cannot be visualized reliably by simple DAPI staining. Due to this constraint, centromeric probes were used to locate the centromere. Two-color FISH, with an ASGR-linked BAC clone (biotin; red signal) and centromeric probes (dig; green signal), was carried out in BC_3, *P. squamulatum*, and *C. ciliaris* (Figure 4, D–F). Interestingly, two centromeric signals were obtained on the chromosome containing the ASGR in BC_3 (Figure 4D) and *P. squamulatum* (Figure 4E). One signal was at the primary constriction of the metacentric chromosome while a second signal was seen at the end of the chromosome, distal to the ASGR-linked BAC signal. In *C. ciliaris*, only a single centromere-related signal, to which the ASGR-linked BAC signal was immediately distal, was observed (Figure 4F).

Location of the ASGR relative to rDNA loci: Two-color FISH was used to locate ASGR-linked BACs (biotin; red signal) in comparison to rDNA loci (dig; green signal). In BC_3, hybridization of metaphase chromosomes with rDNA produced four strong and four weak signals (Figure 4G). *P. squamulatum* showed two major and six minor rDNA signals (Figure 4H). The signal

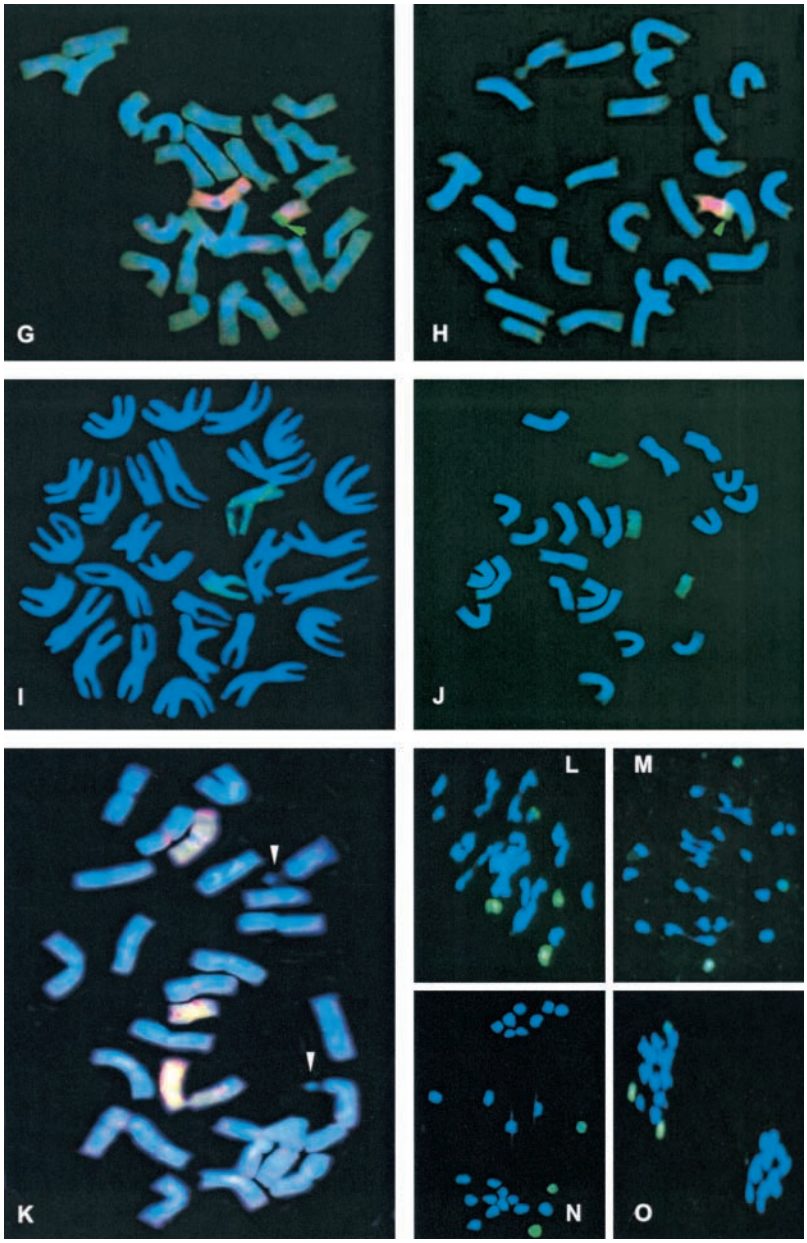

FIGURE 2.—*Continued.*

for the ASGR-linked BAC containing Q8 [p109] was observed on a different chromosome from those showing the rDNA loci in both BC_3 and *P. squamulatum*. *C. ciliaris* showed two major and two minor rDNA signals and, in contrast to BC_3 and *P. squamulatum*, one of the minor rDNA signals shared the chromosome with the ASGR-linked BAC containing ugt197 (c101). The rDNA signal was located distal to the BAC signal (Figure 4I).

The ASGR has no homologous region in *P. squamulatum*: The result of FISH with the ASGR-linked marker mixture and BAC clones as probes against *P. squamulatum*, the apomictic F_1's, and backcrosses showed that a single chromosome carried the terminal ASGR-specific signal. No homologous chromosome could be detected with these probes and hybridization conditions (Figures 2 and 4). Thus, among the 56 chromosomes in *P. squamulatum* (PS26), only 1 chromosome showed the hybridization signal, and F_1 plants with this chromosome were apomictic. No signal from the ASGR-linked markers was observed in any sexual F_1 or backcross plant (Figure 2C; Table 1).

DISCUSSION

Our previous mapping studies have shown that recombination is repressed in the region of the genome that transmits apomixis (OZIAS-AKINS *et al.* 1998). We also have demonstrated that the ASGR is partially hemizygous in nature (OZIAS-AKINS *et al.* 1998; ROCHE *et al.* 1999); thus, during evolution it has undergone consid-

TABLE 1

Results of GISH and FISH with pooled ASGR-linked markers

Generation	Line or plant ID	Phenotype[a]	Tissue used	Probe-DNA[b]	No. of Pg. chrom.[c]	No. of Ps. chrom.[d]	ASGR signal[e]
Parent	PS26 (1/28)[f]	A	Root	PS26 + MM	0	56	+
F_1[g]	124 (3/17)	A	Root	PS26 + MM	14	28	+
	181 (2/13)	A	Root	PS26 + MM	14	28	+
	12 (2/18)	S	Root	PS26 + MM	14	28	−
	105 (2/4)	S	Root	PS26 + MM	14	28	−
BC3	J34 (22/53)	A	Anther	PS26	26	3	NA
	56-1 (1/6)	F	Root	PS26 + MM	26	3	+
	56 (15/87)	A	Root	PS26 + MM	26	3	+
BC5	44-1 (1/4)	A	Root	PS26	26	3	NA
	44-2 (1/5)	A	Root	PS26	27	2	NA
	44-3 (1/13)	A	Root	PS26 + MM	27	2	+
	44-4 (1/12)	S	Root	PS26 + MM	28	1	−
	46-1 (1/1)	F	Root	PS26 + MM	27	2	+
	46-5 (1/8)	A	Root	PS26 + MM	27	2	+
BC6	J35 (12/33)	A	Anther	PS26	26	3	NA
	42-1 (1/5)	F	Root	PS26 + MM	27	2	+
	43-1 (1/5)	F	Root	PS26	26	3	NA
	43-3 (1/5)	F	Root	PS26 + MM	26	3	+
	54 (3/13)	F	Root	PS26 + MM	28	1	+
	62 (2/9)	F	Root	PS26 + MM	28	1	+
BC7	J57-7 (1/5)	F	Anther	PS26		3	NA
	J57 (2/11)	A	Anther	PS26		3	NA
	J60-13 (1/13)	F	Anther	PS26		1	NA
	J64-11 (1/10)	A	Anther	PS26		1	NA
	49-15 (1/3)	S	Root	PS26 + MM	28	0	−
	49 (3/9)	A	Root	PS26	26	3	NA
	58-4 (1/8)	A	Root	PS26	27	2	NA
	58-6 (1/16)	F	Root	PS26 + MM	27	2	+
	60-4 (1/4)	A	Root	PS26	27	2	NA
	60-9 (1/3)	A	Root	PS26	27	2	NA
	60-12 (1/6)	A	Root	PS26 + MM	27	2	+
	61-3 (1/5)	F	Root	PS26	27	2	NA
	61-20 (1/3)	A	Root	PS26 + MM	27	2	+
	61-21 (1/2)	A	Root	PS26	27	2	NA

[a] A, F, and S refer to reproductive phenotypes, respectively, of apomixis (only aposporous embryo sacs observed), facultative apomixis (both aposporous and meiotic embryo sacs observed), and sexual reproduction (only meiotic embryo sacs observed).

[b] DNAs used in FISH and GISH as labeled probes. PS26, genomic DNA from *P. squamulatum*, accession PS26; MM, mixture of ASGR-linked marker DNA.

[c] Number of chromosomes that hybridized with genomic DNA of *P. glaucum*.

[d] Number of chromosomes that hybridized with genomic DNA of *P. squamulatum*.

[e] Presence (+) or absence (−) of hybridization signal revealed by the labeled DNA mixture of ASGR-linked markers. NA, not applicable.

[f] Numbers in parentheses indicate the number of plant(s) (before slash) and spreads (after slash) investigated.

[g] F_1 of tetraploid *P. glaucum* × *P. squamulatum* (Ozias-Akins *et al.* 1998).

erable sequence and perhaps structural divergence from orthologous and even allelic regions. In this article we show that transmission of this single distinct chromosome is sufficient to confer apomixis in backcrosses with a sexual species. Additional evidence for hemizygosity and other structural features of the ASGR-carrier chromosome from two apomictic species are reported in this work and their potential to impact recombination is discussed.

One chromosome with the ASGR is sufficient for the expression of apomixis: In this study, hybridization of multiple ASGR-linked probes to several apomictic genotypes (species, crosses, and backcrosses) detected a single hybridizing chromosome in each of the plants tested. Previous mapping of apomixis-linked molecular markers from an apomictic BC_3 line to a BC_4 population strongly suggested that a single chromosome was necessary and sufficient for expression of the trait (Ozias-

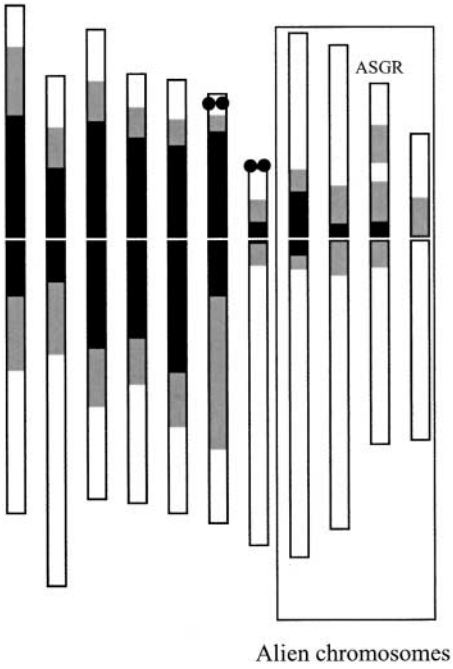

FIGURE 3.—Ideogram of BC_3 based on five chromosome spreads. Black and gray areas indicate levels of high and moderate condensation, respectively, as determined from DAPI fluorescence. The threshold for high condensation was based on the appearance of the black region on chromosome 7. The threshold for moderate condensation was based on the appearance of the region on the short arm of the ASGR-carrier chromosome.

AKINS *et al.* 1993). Similarly, apomixis was mapped as a single-dose allele in *P. squamulatum* (OZIAS-AKINS *et al.* 1998). The uniqueness of ASGR-linked SCAR sequences (~1 kb in size) had been previously shown by Southern blots where hybridization and segregation data led to their description as hemizygous sequences (OZIAS-AKINS *et al.* 1998; ROCHE *et al.* 1999). In this study, FISH probes consisting of entire ASGR-linked BAC clones (80- to 100-kb inserts) also resulted in the observation of hemizygosity where, in all cases, BAC-FISH signal was detected on only a single chromosome in apomictic plants. The ASGR-carrier chromosome, which was ultimately identifiable by morphological characteristics and multiple FISH probes, was always absent from sexual plants even though these plants could carry other chromosomes that hybridized with *P. squamulatum* DNA by GISH. Although the lack of any detectable signal from ASGR-linked BACs to a homologous chromosome in backcrosses was not unexpected, given that the majority of chromosomes were derived from the sexual recurrent parent, *P. glaucum*, we anticipated that additional hybridization signals of the ASGR-linked BACs to chromosomes in *P. squamulatum* or *C. ciliaris* may occur. Both *P. squamulatum* and *C. ciliaris* are polyploid; thus more than two homologous or homeologous chromosomes containing allelic sequences could have hybridized with the BAC clones. In *Arabidopsis thaliana*, a contig of BAC clones produced allelic hybridization signals on a pair of chromosomes, whereas the same probe produced multiallelic signals in *Brassica rapa* (JACKSON *et al.* 2000). Similarly, a dihaploid potato clone from tetraploid potato always showed signals on a pair of somatic chromosomes when probed with BAC clones from a potato BAC library (SONG *et al.* 2000).

Our FISH results confirm our earlier genetic and molecular mapping studies, which concluded that apomixis was controlled by a single, dominant "locus" (a locus being defined here as an inherited unit, not necessarily a gene). Since the ASGR does not have a strictly colinear region on the "sexual" homologs in *P. squamulatum*, it should behave genetically like a dominant, single gene. This inheritance pattern also is consistent with other recent molecular genetic studies, which have contributed to the emergence of a predominant hypothesis that apomixis is regulated by one or two, usually dominant loci (GROSSNIKLAUS *et al.* 2001). Given the absolute correlation between the presence of the ASGR-carrier chromosome and apomictic reproduction, it seems likely that there are apomixis-specific factors in the ASGR, which underlie certain developmental pathways

TABLE 2

Characteristics of the chromosomes in BC_3

Chromosome no.	Length (μm)	SD	Arm ratio (*L*/*S*)	SD	Note
1	10.89	1.54	1.21	0.25	
2	10.88	1.64	2.03	0.67	
3	10.24	1.71	1.37	0.35	
4	9.26	1.21	1.62	0.27	
5	9.08	1.34	1.75	0.37	
6	9.23	1.29	2.01	0.37	rDNA
7	8.03	1.21	4.38	0.89	rDNA satellite
Alien 1	11.30	1.61	1.59	0.36	
Alien 2	10.71	1.11	1.66	0.39	
Alien 3	7.80	1.10	1.34	0.19	ASGR
Alien 4	6.61	1.43	1.96	0.61	

Chromosomes 1–7 are present in two to four copies each, whereas each alien chromosome is present as a single copy.

TABLE 3

ASGR-linked BAC clones used for the present FISH study

Origin of BAC library	SCAR marker content[a]	BAC clone ID	Apomict probed
Polyhaploid	Q8M	p109	BC_3 *P. squamulatum*
	Ugt197	p201	BC_3
		p202	*P. squamulatum*
		p203	
		p205	
		p207	
	A10H	p303	BC_3
	A14M	p001	BC_3
	O7M	p503	BC_3
C. ciliaris	Q8M	c001	*C. ciliaris*
	Q8M	c013	*C. ciliaris*
	Ugt197	c101	*C. ciliaris*

[a] All BACs were PCR positive for the respective SCAR markers.

that allow nucellar cells to form aposporous embryo sacs. Alternatively, the ASGR could place a group of reproduction-related genes in a different structural context through an effect on chromatin remodeling, thereby changing gene expression patterns.

While a single chromosome from *P. squamulatum* is sufficient for the expression of apomixis, we have noted considerable variation in the degree of apomixis (as measured by progeny analysis) among various aposporous backcross lines ranging in chromosome number from 27 to 29 (Hanna *et al.* 1993). These exact materials were not available for FISH analysis, and only small numbers of progeny of one-, two-, and three-chromosome addition/substitution backcross lines from this study were analyzed. Preliminary observations have shown that the BC_7 lines in this study with one, two, or three alien chromosomes can have as few as 40–60% or as many as 85–100% of the pistils producing aposporous embryo sacs. Progeny analysis in these materials generally indicates a correlation between the frequency of embryo sac type and reproductive mode. The facultativeness we have observed also could be affected by environment (W. W. Hanna, personal observations), although there is little published evidence for such effects in Pennisetum (Hussey *et al.* 1991). Genetic modifiers of apomixis have been reported to exist in Hieracium (Koltunow *et al.* 2000), and it may eventually be necessary to understand the role of such modifiers in the penetrance of the trait if it is to be utilized in crop plant breeding and genotype maintenance. To carefully analyze the penetrance of the trait in different backcross lines of Pennisetum, a multiyear study that includes both greenhouse and field environments, plus embryo sac clearing and progeny analysis, will need to be conducted.

Structural features of the ASGR-carrier chromosome: Four major and four minor rDNA signals were observed in BC_3, which has a tetraploid pearl millet background. These results are consistent with earlier reports of two major and two minor rDNA signals for diploid *P. glaucum* (Martel *et al.* 1996; Liu *et al.* 1997). *P. squamulatum* exhibited two major and six minor rDNA sites, while *C. ciliaris* contained two major and two minor rDNA sites. The locations of rDNA, with respect to the ASGR, differentiated *C. ciliaris* from *P. squamulatum*. Ribosomal DNA sites have been shown to be mobile in species such as wheat and rice (Dubcovsky and Dvorak 1995; Shishido *et al.* 2000). Translocation of distal regions has been suggested as the origin of the new rDNA loci in some species (Arnheim 1983; Badaeva *et al.* 1996). The mechanisms responsible for the apparent mobility of rDNA are not clear and, although transposition or translocation may be involved, it also is possible that cryptic rDNA sites could be amplified by unequal crossing over (Dubcovsky and Dvorak 1995). Comparing the rDNA location with the ASGR in other apomictic Pennisetum species may provide information about the evolution of the ASGR in these plants.

An abundant 160-bp cloned repeat from *P. squamulatum* was shown to localize to the centromeres of pearl millet and *P. squamulatum* as well as to the end of the ASGR-carrier chromosome. The 140-bp repeat from pearl millet shares significant sequence similarity with *P. squamulatum* repeats (140 and 160 bp), even though there is an additional repeat unit structure in *P. squamulatum* (Ingham *et al.* 1993). According to Ingham *et al.* (1993), the smaller repeat family likely diverged from the larger repeat unit by a deletion.

Recombination in the ASGR: Multiple hypotheses to explain the basis of repressed recombination in the ASGR can be found in the literature (Grimanelli *et al.* 1998; Ozias-Akins *et al.* 1998; Grossniklaus *et al.* 2001; Roche *et al.* 2001). A prerequisite for recombination is pairing and synapsis of homologous chromosomes. Ozias-Akins *et al.* (1998) and Roche *et al.* (2001) suggested that the apospory locus could be located on a mini- or a B chromosome devoid of any pairing homolog. Although there are reports of B chromosomes in *P. squamulatum* (Sindhe 1976), and indeed the accession we used in this study contained 56 chromosomes, which presumably reflects the presence of two B chromosomes in addition to the normal hexaploid complement of 54 chromosomes, no relationship has been established between the aposporous phenotype and the presence of a B chromosome. Obligate apomixis also has been observed in accessions of *P. squamulatum* containing only 54 chromosomes (Dujardin and Hanna 1984b). Furthermore, the ASGR-carrier chromosome does not appear to segregate strictly as a univalent (although univalents have been reported in *P. squamulatum*; Sindhe 1976; Dujardin and Hanna 1984b), because amplified fragment length polymorphism markers

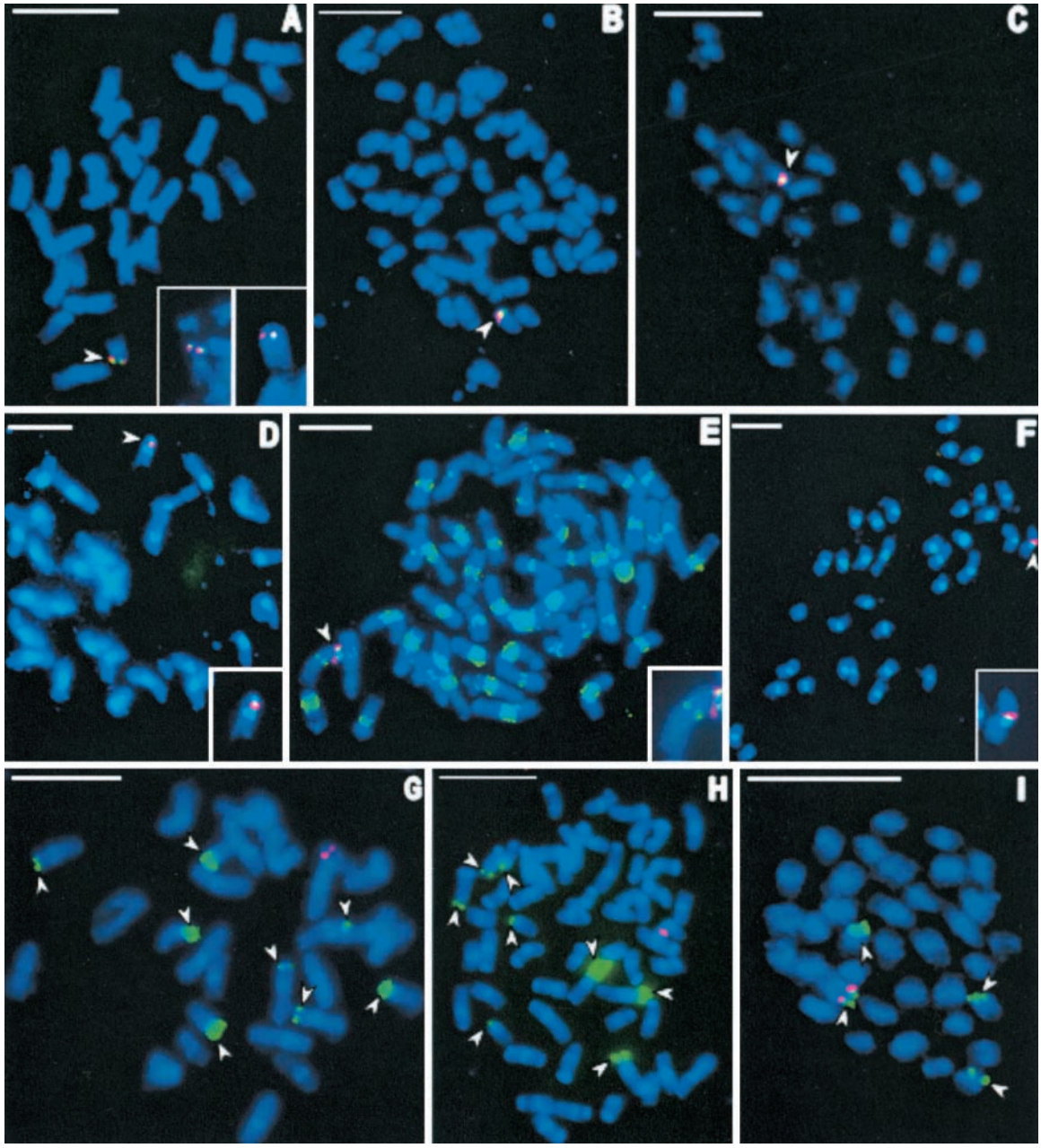

FIGURE 4.—Localization of ASGR-linked BAC clones on metaphase chromosomes by dual-target mapping with two BACs (A–C), centromeric probes (D–F), and ribosomal DNA (G–I). In A–C, hybridizing sites on the ASGR-carrier chromosome are marked with arrowheads. Images in insets show enlarged ASGR-carrier chromosome. (A) BC_3 hybridized with BAC clones p203 (red) and p109 (green); images in inset show signals for p207 (red)/p001 (green) and p303 (green)/p503 (red). (B) *P. squamulatum* hybridized with p207 (red) and p109 (green). (C) *C. ciliaris* hybridized with c001 (green) and c101 (red). Overlapping signals in A–C are yellow. (D) BC_3 hybridized with p205 (red) and 160-bp *Kpn*I centromere repeat (green). (E) *P. squamulatum* hybridized with p208 (red) and a 160-bp *Kpn*I centromere repeat (green). (F) *C. ciliaris* hybridized with c013 (red) and a BAC containing centromere repeats (green). (G) BC_3 hybridized with p109 (red) and rDNA (green, arrowheads). (H) *P. squamulatum* hybridized with p109 (red) and rDNA (green, arrowheads). (I) *C. ciliaris* hybridized with c101 (red) and rDNA (green, arrowheads). Bars, 10 μm.

displaying recombination are linked with, but outside of, the ASGR (Z. B. CHEN, W. W. HANNA and P. OZIAS-AKINS, unpublished data).

Even though the entire ASGR-carrier chromosome does not strictly behave as a B chromosome, the ASGR itself does display characteristics, such as hemizygosity, that set it apart from other regions of the genome. The unusual features of the ASGR could be explained by a combination of male-only transmission following several potential generative processes: (i) introgression of a divergent chromosomal fragment through hybridization such as has been observed through artificial hybrid-

ization of sugar beet with wild relatives to transfer nematode resistance (Schmidt *et al.* 1997), (ii) the invasion of retrotransposons and their subsequent amplification as in maize knobs (Ananiev *et al.* 1998), (iii) the *de novo* assembly of satellite DNA families into a distinct heterochromatic domain with "an ostensibly foreign origin" (Langdon *et al.* 2000), (iv) the fusion of otherwise free supernumerary chromatin to an autosomal chromosome as previously described in insects (Araujo *et al.* 2001), or (v) targeted deletion as part of a selfish maintenance mechanism similar to gametocidal genes (Ogihara *et al.* 1994). Any of these mechanisms could lead to sequence dissimilarity among homologs and interfere with recombination.

Another hypothesis for repressed recombination has been that the apospory "locus" could be located near the centromere of a chromosome (Ozias-Akins *et al.* 1998). Reduced recombination already has been shown around the centromeric regions of various organisms (Tanksley *et al.* 1992; Davis *et al.* 1994; Sherman and Stack 1995; Round *et al.* 1997; Mahtani and Willard 1998) and could be due, at least in part, to the formation of centromeric heterochromatin (Khush and Rick 1968; Rick 1972; Copenhaver and Preuss 1999). Data from this study support proximity to a centromere as a possible cause of low recombination in the ASGR, at least for *C. ciliaris*. Even for the ASGR in *P. squamulatum*, the detection of additional centromeric repeats at the end of the ASGR-carrier chromosome could have consequences on recombination. We do not have any evidence for retention of functionality of the terminal centromeric sequences (such a dicentric chromosome probably would not have survived the backcrossing program with two centromeres of equal strength), although the presence of terminal centromeric sequences alone possibly could alter adjacent chromatin structure and lead to reduced recombination.

A third hypothesis for repressed recombination at the ASGR was the presence of a heterozygous inversion (Ozias-Akins *et al.* 1998). Heterozygous inversions are well known to influence apparent recombination rates since a single crossover within an inversion loop formed by the pairing of a paracentric inversion with a normal chromosome would lead to the formation of duplications and deficiencies, including dicentric and acentric chromosomes, neither of which is likely to be transmitted (Burnham 1962; Schulz-Schaeffer 1980). Such chromosomal aberrations usually result in reduced fertility, although we have observed relatively high pollen viability in locally tested accessions of *P. squamulatum* (82%; Dujardin and Hanna 1984b) in contrast to 56% sterility observed by Sindhe (1976). However, paracentric inversions can also cause localized asynapsis (lack of pairing) or desynapsis (precocious separation of the paired chromosomes) without leading to chromosomal aberrations (Schulz-Schaeffer 1980) but still resulting in suppressed recombination at the ASGR. Since the location of the ASGR signal and chromosome morphology were similar in both BC_3 and *P. squamulatum*, no major rearrangement appears to have survived meiosis during the transfer of the chromosome from *P. squamulatum* to BC_3. However, a comparison of the position of the ASGR and associated centromeric sequences between *P. squamulatum* and *C. ciliaris* does suggest that an inversion probably occurred during evolutionary separation of these two species.

The physical localization data provided by FISH offers explanations for repressed recombination at the ASGR. Repressed recombination makes it possible that apospory is controlled, not by a single gene, but rather by two or more genes that are maintained as an intact genetic unit. Gametophytic apomixis requires at least two universal components: formation of unreduced embryo sacs and the capacity for parthenogenetic development of egg cells. It seems unlikely that a single gene controls both of these characters, although models have been proposed for a single master regulator that could cause the precocious expression of certain genes in apomicts that are normally expressed only after meiosis in sexual plants (Peacock 1992). The idea of the ASGR representing a haplotype with two or more tightly linked genes becomes more appealing in the wake of recent evidence that diplosporous apomixis is governed by more than one locus, where diplospory and parthenogenesis clearly can be uncoupled in some species (Tas and van Dijk 1999; van Dijk *et al.* 1999; Noyes and Rieseberg 2000). Many examples of multigene or complex loci have been found in both plants and animals. Coadapted gene complexes include examples such as the self-incompatibility loci of Brassica, the *t* haplotype in mouse, and sex chromosomes. Distinct haplotypes emerge in these cases where genes remain linked in phase by the lack of regional intrachromosomal recombination.

Our goal is the isolation of genes for aposporous apomixis that have been mapped to a single ASGR-carrier chromosome, which is sufficient for the expression of apomixis in the background of tetraploid pearl millet. Although this goal is likely beyond our immediate reach, further elucidation of the finer structure of the ASGR and studies into the possible sequence rearrangements at the locus will give more insights into the evolution of apospory in the Pennisetum/Cenchrus complex. To achieve our long-term goal, fragmentation of the ASGR-carrier chromosome and radiation hybrid mapping may be essential for dissection of the detailed structure of the ASGR to the minimum unit required for aposporous reproduction. Such an experimental approach also should generate materials that could shed light on the relevance of the chromosomal context of this hemizygous region for the expression of apomixis.

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