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Comparative Physical Mapping of the Apospory-Specific Genomic Region in Two Apomictic Grasses: *Pennisetum squamulatum* and *Cenchrus ciliaris*

Shailendra Goel,* Zhenbang Chen,*[,1,2] Yukio Akiyama,*[,1,3] Joann A. Conner,* Manojit Basu,*[,4] Gustavo Gualtieri,*[,5] Wayne W. Hanna[†] and Peggy Ozias-Akins*[,6]

**Department of Horticulture, University of Georgia Tifton Campus, Tifton, Georgia 31793-0748 and [†]Department of Crop and Soil Sciences, University of Georgia Tifton Campus, Tifton, Georgia 31793-0748*

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ABSTRACT

In gametophytic apomicts of the aposporous type, each cell of the embryo sac is genetically identical to somatic cells of the ovule because they are products of mitosis, not of meiosis. The egg of the aposporous embryo sac follows parthenogenetic development into an embryo; therefore, uniform progeny result even from heterozygous plants, a trait that would be valuable for many crop species. Attempts to introgress apomixis from wild relatives into major crops through traditional breeding have been hindered by low or no recombination within the chromosomal region governing this trait (the apospory-specific genomic region or ASGR). The lack of recombination also has been a major obstacle to positional cloning of key genes. To further delineate and characterize the nonrecombinant ASGR, we have identified eight new ASGR-linked, AFLP-based molecular markers, only one of which showed recombination with the trait for aposporous embryo sac development. Bacterial artificial chromosome (BAC) clones identified with the ASGR-linked AFLPs or previously mapped markers, when mapped by fluorescence *in situ* hybridization in *Pennisetum squamulatum* and *Cenchrus ciliaris*, showed almost complete macrosynteny between the two apomictic grasses throughout the ASGR, although with an inverted order. A BAC identified with the recombinant AFLP marker mapped most proximal to the centromere of the ASGR-carrier chromosome in *P. squamulatum* but was not located on the ASGR-carrier chromosome in *C. ciliaris*. Exceptional regions where synteny was disrupted probably are nonessential for expression of the aposporous trait. The ASGR appears to be maintained as a haplotype even though its position in the genome can be variable.

APOMIXIS is a naturally occurring mode of asexual propagation in angiosperms, where it is defined as clonal propagation through seeds. This asexual mode of reproduction exists in numerous plant families, but is most frequent in the eudicot families Rosaceae and Asteraceae and in the monocot family Poaceae (Richards 1986; Asker and Jerling 1992). In addition to the theoretical interest of better understanding the relationship between sexual and apomictic modes of plant reproduction, the ability to manipulate apomixis has vast potential for practical application. The main agronomic benefit would be the fixation of heterosis (Hanna 1995; Koltunow *et al.* 1995; Grossniklaus *et al.* 1998). The potential economic value of apomixis technology for hybrid rice production was estimated to exceed US $2.5 billion per annum (McMeniman and Lubulwa 1997). Considering this evaluation, the recent trend of increasing interest in the underlying mechanism of apomixis is hardly surprising.

Apomixis has two basic forms: sporophytic apomixis and gametophytic apomixis. In sporophytic apomixis, also called adventitious embryony, embryos are directly initiated from nongenerative cells of the ovule. Gametophytic apomixis has two forms: apospory and diplospory. In apospory, embryo sacs develop from nucellar cells, while in diplospory the generative cell undergoes mitosis to form an embryo sac. In both cases, a chromosomally unreduced cell forms a megagametophyte in which the egg cell parthenogenetically gives rise to an embryo with a genetic constitution identical that of to the female parent.

Recent studies have shown that diplosporous apomixis is controlled by independent loci for diplosporous embryo sac formation and parthenogenesis in apomictic Taraxacum species (van Dijk *et al.* 1999) and in *Erigeron annuus* (Noyes and Rieseberg 2000). Conversely, apospory has been shown to be inherited as a

[1]These authors contributed equally to this work.

[2]*Present address:* Department of Crop and Soil Sciences, Georgia Experiment Station, Griffin, GA 30223.

[3]*Present address:* Venture Business Laboratory, Center of Advanced Science and Innovation, Osaka University, Osaka 565-0871, Japan.

[4]*Present address:* Division of Plant Science, School of Biosciences, Sutton Bonnington Campus, Loughborough, Leicestershire, LE12 5RD, United Kingdom.

[5]*Present address:* School of Integrative Biology, Faculty of Biological and Chemical Sciences, The University of Queensland, Brisbane, QLD 4072, Australia.

[6]*Corresponding author:* Department of Horticulture, University of Georgia Tifton Campus, 2356 Rainwater Rd., Tifton, GA 31794.
E-mail: pozias@uga.edu

TABLE 1

Primers for different SCAR markers used in this study

Primer pair sequence		Source of sequence	Annealing temperature	Fragment size (bp)
Forward primer	Reverse primer			
5′-TTACCAGGTCGCCATAAGCAAG-3′	5′-TGTTCTGACCAACCTGCTCC-3′	AL606627	58	873
5′-GATTGTGACGTTGAAGAAAA-3′	5′-TAACCATGCCTCCACTATGA-3′	BAC C1000	49	378
5′-ACTATCCTCAGAAAAGATGTAACAG-3′	5′-GATTTGCTGTTGGAGGGAAT-3′	BAC C1000	52	350
5′-AGAATTTTAGATGGCTTCTG-3′	5′-CACCAAAAAACTGTAATGTC-3′	BAC C1000	49	304
5′-ATTGGCAGAATCAAATGCCTACATAACTC-3′	5′-TCATGGATCAGGATCTTACTCGACA-3′	BAC P1200	58	800

monogenic, dominant Mendelian trait (Grimanelli *et al.* 2001; Grossniklaus *et al.* 2001), and only in the case of *Poa pratensis* has aposporous apomixis been shown to have recombination between loci governing apospory and parthenogenesis (Albertini *et al.* 2001).

Aposporous apomixis in *Pennisetum squamulatum* and *Cenchrus ciliaris* (buffelgrass, syn. *P. ciliare*) is conferred by an apospory-specific genomic region (ASGR) that is a substantial portion of a chromosome arm and hemizygous in nature (Goel *et al.* 2003; Ozias-Akins *et al.* 2003; Akiyama *et al.* 2004, 2005). There has been no detectable recombination in the ASGR (Ozias-Akins *et al.* 1998; Roche *et al.* 1999); however, in this work we identify a single recombinant amplified fragment length polymorphism (AFLP) marker and use this along with other ASGR-linked AFLP markers for further investigation of physical distances delineated by markers and the recombination behavior of the ASGR. Furthermore, macrosynteny between the two species *P. squamulatum* and *C. ciliaris* was studied by carrying out comparative fluorescence *in situ* hybridization (FISH) analysis of the ASGR.

MATERIALS AND METHODS

Mapping populations and trait screening: Two separate populations of 94 and 99 F_1 progeny from crosses between *P. glaucum* (induced tetraploid, $2n = 28$) and *P. squamulatum* (PI 319196, $2n = 56$) were used for AFLP analysis. The first population was composed of 94 F_1 plants (46 sexual and 48 apomicts) and was a subset of the mapping population used by Ozias-Akins *et al.* (1998). This population was used for whole-genome mapping. The second population of 99 F_1 plants (45 sexual, 45 apomicts, and 9 classified for markers but not phenotype) was characterized for mode of reproduction by clearing of ovules in methyl salicylate (Young *et al.* 1979) and for marker phenotype using two ASGR-linked sequence-characterized amplified region (SCAR) markers (UGT197 and Q8M) reported by Ozias-Akins *et al.* (1998). This population was used for the confirmation of ASGR-linked AFLP markers and their further analysis since plant material for all individuals of the first population was no longer available. An F_1 population of *C. ciliaris* that previously had been described as segregating for apomixis and sexual reproduction (Roche *et al.* 1999) was used to identify SCAR markers cosegregating with apomixis on the basis of the sequence from putative ASGR-linked BACs.

Amplification of SCARs: SCAR markers UGT197 and Q8M were previously described (Ozias-Akins *et al.* 1998). Primer sequences for additional SCAR markers are given in Table 1 along with the source of primer sequence, size of amplification product, and annealing temperature used for amplification.

AFLP analysis: AFLP analysis was carried out following the protocol of the ABI PRISM fluorescent dye-labeled AFLP kit for large genomes (Applied Biosystems, Foster City, CA) with some modifications as described below. Digestion and ligation were carried out in a single reaction for 3 hr at 37° in a reaction volume of 11 μl, including 500 ng genomic DNA, 50 mM NaCl, 0.1 mg/ml BSA, 8 units *Eco*RI, 1 unit *Mse*I (New England Biolabs, Beverly, MA), 0.1 μM of *Eco*RI adapter, and 0.5 μM of *Mse*I adapter, 1× T4 DNA ligase buffer, and 0.3 unit T4 DNA ligase (Promega, Madison, WI). Digestion and ligation were followed by preselective amplification using adenine (A) and cytosine (C) as preselective nucleotides for *Eco*RI primer ends (*Eco*RI + A) and *Mse*I primer ends (*Mse*I + C), respectively. For subsequent selective amplifications, a 40× dilution of preamplified products was found to reduce nonspecific background on gel images. Selective amplifications were carried out with three nucleotide extensions (*Eco*RI + 3/*Mse*I + 3) of each adapter under a stringent touch-down temperature profile as described in the manufacturer's protocol. The PCR reactions were then loaded on 4% denaturing polyacrylamide sequencing gels and run on an ABI PRISM 377 DNA sequencer. Gel images were generated with GeneScan 3.1 software (Applied Biosystems).

Initially, 10 F_1 plants (5 apomicts and 5 sexual plants) and the two parents were used to test all 64 primer pairs supplied with the kit for amplification of informative AFLPs. Primers that produced the largest number of informative fragments were later synthesized and 5′ end labeled with the fluorescent dye FAM (Invitrogen, Carlsbad, CA). Twenty-six selected primer combinations were used for screening the mapping population of 94 F_1 plants plus two parents (Table 2).

Bands on the gel image were scored manually as 1 (band present) or 0 (band absent). Only the loci that were present in *P. squamulatum* and absent in pearl millet and were segregating among the progeny were considered for linkage analysis. Each informative polymorphic AFLP fragment was identified by the combination of a two-letter prefix designating the primer combination and a number indicating the estimated molecular weight of the fragment (Table 2). For each locus, the ratio of the number of individuals scored as positive or negative for a locus was tested with χ^2 for a 1:1 fit at $P < 0.05$ and d.f. = 1. Loci that fit a 1:1 segregation ratio were considered to be single-dose AFLP markers since a 1:1 ratio would be expected in a testcross (Wu *et al.* 1992).

Linkage maps were generated with Mapmaker/Exp version 3.0 (Lander *et al.* 1987). Markers were first sorted by using the group command at a LOD score threshold of 5 and a recombination fraction threshold of ≤0.37. The LOD score

subsequently was reduced to 4 and 3 while maintaining a maximum recombination fraction ≤0.37 to find the possible additional, but statistically less well supported, linkages. Orders of marker loci were determined with multi-point comparisons using the standard commands. When no starting point could be found, two-point comparisons were used to determine the order.

Gel purification of AFLP fragments: To recover an AFLP fragment from a gel, unlabeled *Eco*RI + 3 primer was used for selective amplification. The AFLP reaction was loaded onto a 5% denaturing acrylamide gel and subjected to electrophoresis, and the gel was silver stained using the Silver Sequence DNA sequencing system (Promega) following the manufacturer's instructions. The gel slice of interest was excised, placed in 20 μl water, frozen at −80° for 10 min, incubated at 37° for 10 min, and finally centrifuged for 10 min at 17,000 × *g* in a microcentrifuge. A 2-μl aliquot of the supernatant was used for PCR with the same primers and conditions used for selective amplification. The amplified product was subjected to two more cycles of elution and amplification as described above to ensure that the product was free of contamination from bands of similar size. Eluted bands were labeled by PCR incorporation of ^{32}P-labeled dCTP using the corresponding primers and PCR conditions similar to selective amplification.

Isolation of BACs containing AFLP markers: Construction of BAC libraries from two apomictic genotypes (a polyhaploid from *P. glaucum* × *P. squamulatum* and buffelgrass) has been reported previously (Roche *et al.* 2002). Screening for ASGR-linked BAC clones containing markers UGT197 and Q8M and their further characterization also has been described (Roche *et al.* 2002; Goel *et al.* 2003; Akiyama *et al.* 2004).

To identify BACs containing mapped AFLP markers, AFLP was carried out on a subset of clones (39,936 of 118,272) from the polyhaploid BAC library (Roche *et al.* 2002). Two to three 384-well microtiter plates containing the BAC clones were pooled together to obtain 42 pools (representing a total of 104 384-well plates). The 384-well plates were grown separately before pooling to provide equal representation of clones in the pooled culture. DNA extraction from each pool was carried out following the standard alkaline-lysis method for plasmid isolation (Sambrook *et al.* 1989). Approximately 350–500 ng of the plasmid DNA was subjected to AFLP analysis using similar conditions as described above for plant DNA, including preamplification. After selective amplification, the reactions were run on a gel along with a selective amplification reaction from an apomictic F_1 individual. To identify the single positive BAC from a pool, colony blots of the positive pools were hybridized with the labeled AFLP marker.

Individual plates from BAC pools showing the presence of a particular AFLP marker were stamped onto colony/plaque screen hybridization transfer membrane (Perkin-Elmer, Boston) using a 386-pin replicator (Boekel Scientific, Feasterville, PA) and grown overnight on LB–agar plus 15 μg/ml chloramphenicol. On the following day, colony blots were prepared by incubating membranes sequentially on 10% SDS (w/v), denaturing solution (1.5 m NaCl, 0.5 m NaOH), and neutralizing solution (0.5 m Tris.Cl pH 7.4, 1.5 m NaCl) for 5 min each. Blots were washed in 2× SSPE, air dried, and hybridized with the corresponding eluted and ^{32}P-labeled AFLP bands.

FISH: Mitotic chromosome preparations were made according to Goel *et al.* (2003) while pachytene chromosomes were prepared according to Akiyama *et al.* (2004). Pretreatment, hybridization, probe detection, and image capture were accomplished according to Goel *et al.* (2003). Unless stated, no blocking DNA was used in hybridizations. Image analysis was carried out using ImageJ 1.34a (http://rsb.info.nih.gov/ij/) and CHIAS (Kato and Fukui 1998). Composite images and image enhancement were carried out with Adobe Photoshop version 5.0.

Contig assembly and chromosome walking: All ASGR-linked BAC clones were subjected to fingerprinting contig analysis (FPC) including BAC clones that had been isolated by chromosome walking (Gualtieri *et al.* 2006). BAC DNAs were digested with *Hin*dIII and run for 16 hr on 1% agarose gels in 1× TAE at 10° and 85 V. Gels were stained with SYBR Green (Cambrex) and scanned using the STORM gel and blot imaging system (Amersham Biosciences, Piscataway, NJ). Band calling and contig building were accomplished using Image (version 3.10, Sanger Centre, UK) and FPC (version 4.6, Clemson University Genomics Institute). FPC analysis parameters were set at a tolerance of 7 and stringency level of 10^{-12}. Contigs 1 and 16 (Table 3) were coalesced together on the basis of RFLP data presented elsewhere (Gualtieri *et al.* 2006).

RESULTS

AFLP markers: Sixty-four primer pairs (8 *Eco*RI + 3 × 8 *Mse*I + 3) were screened to determine the most informative combinations. Twenty-six primer combinations produced reproducible and polymorphic bands between the two parents as well as among 94 F_1 progeny (Table 2), the number ranging from a low of 36 (ACG/CAT) to a high of 116 (ACT/CTA) with an average of 78.8 bands. Polymorphic bands composed up to 90.9% of the total amplified bands (Table 2). AFLP fragments that fit a 1:1 ratio according to the χ^2 for goodness-of-fit test ($P < 0.05$, d.f. = 1) were considered to be single-dose AFLP markers and were included in the data set used for mapping. Among these polymorphic bands, 1–30 loci per AFLP primer combination qualified as single-dose AFLP markers, which was only 12.4% of the total number of polymorphic bands. Multiple-dose markers were not included for mapping because pairing relationships among *P. squamulatum* chromosomes have not been fully determined. Bands shared by both *P. squamulatum* and *P. glaucum* made up 9% of the total number of amplified bands.

A total of 230 single-dose markers were grouped, at a LOD score of 5, into 48 linkage blocks with 73 unlinked markers. When the LOD score was reduced to 3, 23 previously unlinked markers were added to the map, and the number of linkage groups was reduced from 48 to 44. A linkage map constructed at LOD 3 and a maximum recombination fraction of 0.37 had a total length of 2600 cM with an average distance of 14.3 cM between markers. Only low-density coverage of the 56-chromosome genome was achieved with 180 single-dose AFLP markers whereas 50 markers, or 21.7% of the total, were left unlinked. The mapped AFLP markers showed primarily random distribution. The objective of finding additional markers linked with apomixis was met since the largest cluster that contained seven markers and was positioned on the longest linkage group also cosegregated with the trait of aposporous embryo sac development and the previously characterized SCAR UGT197 (Ozias-Akins *et al.* 1998). No other cluster of

TABLE 2

Primer combinations and summary of data generated with each combination

ID	Primer combination	Amplified bands[a]	Shared bands[b]	M-D bands[c]	S-D bands[d]	Mapped bands	% of M/A[e]
Pa	AAC/CTA	91	6	69	16	13	14.3
Pb	ACA/CAT	71	12	52	7	7	9.8
Pc	ACC/CAA	68	4	55	9	8	11.8
Pd	ACG/CAG	93	20	63	10	9	9.7
Pe	AGG/CAT	85	22	52	11	8	9.4
Pf	ACT/CAT	85	12	64	9	7	8.2
Pg	ACC/CAT	65	3	58	4	2	3.1
Ph	AGG/CAG	88	2	81	5	1	1.1
Pi	AGG/CTA	91	4	79	8	6	6.6
Pj	AGG/CTC	79	6	62	11	7	8.9
Pl	ACG/CAT	36	2	31	3	3	8.3
Pm	AAC/CAG	55	7	44	4	3	5.4
Pn	ACG/CTC	54	7	43	4	3	5.6
Po	ACA/CTC	78	11	61	6	4	5.1
Pp	ACT/CAA	74	7	66	1	1	1.4
Pq	ACA/CAA	95	13	66	16	15	15.8
Pr	AGG/CAA	88	2	78	8	6	6.8
Ps	ACT/CAG	74	1	65	8	7	9.5
Pt	ACT/CTC	60	3	47	10	7	11.7
Pu	ACT/CTA	116	8	78	30	24	20.7
Pv	ACA/CAG	86	11	64	11	8	9.3
Pw	ACG/CTA	88	8	65	15	14	15.9
Px	ACG/CAA	62	6	45	11	9	14.
Py	AGC/CTA	90	1	85	4	4	4.4
Pz	AGC/CAG	89	3	78	8	5	5.6
pA	AGC/CAA	89	6	82	1	1	1.1
Total	26	2050	187	1633	230	182	
Average		78.8	7.2(9.1)[f]	62.8(79.7)[f]	8.8(11.2)[f]	7	8.9

[a] Total number of bands amplified with each primer combination.
[b] Bands shared by both *P. squamulatum* and *P. glaucum.*
[c] Multiple-dose bands segregating at a ratio statistically >1:1.
[d] Single-dose bands segregating at a ratio of 1:1 ($P < 0.05$).
[e] The percentage of mapped bands out of total amplified bands.
[f] The number in parentheses refers to the percentage of total amplified bands.

>2 markers was found on the remaining 43 linkage groups. The seven clustered apomixis-linked markers (pu70, pa502, pv600, pw298, py503, px299, and pa265) were totally linked with apospory and composed 3.0% of the total single-dose AFLP markers. The only recombining flanking marker for the target trait (apospory) was pq355. This marker was linked to apospory at a distance of 2.02 cM.

BAC clones were isolated for four of the ASGR-linked AFLP markers (pa265, pq355, px299, and py503) by BAC AFLP and colony hybridization (Table 3). All BAC(s) were further confirmed for the presence of the corresponding AFLP marker when the correctly sized band on *Eco*RI + *Mse*I BAC blots hybridized with the labeled AFLP band.

Contig development: A total of 100 ASGR-linked BAC clones isolated from polyhaploid and buffelgrass libraries (Roche *et al.* 2002; Gualtieri *et al.* 2006) were fingerprinted at 10^{-10} and 10^{-12} stringency levels. At a stringency level of 10^{-10}, 86 BAC clones were present in various contigs and 14 were singletons whereas at stringency level 10^{-12}, 73 BAC clones were present in various contigs and 27 BAC clones were singletons. Results for contig formation at stringency 10^{-12} are summarized in Table 3 where each row represents a contig except the rows marked "singleton." The italic rows are contigs that contain BAC clones from both buffelgrass and *P. squamulatum.*

The most BAC clones were linked to ASGR SCAR markers Q8M (14 from the buffelgrass library and 8 from the polyhaploid library) and UGT197 (13 for the buffelgrass library and 8 from the polyhaploid library). The FPC assembled contigs from both of these marker classes, and the related clones obtained through chromosome walking (Gualtieri *et al.* 2006), contained BAC clones from both *C. ciliaris* and *P. squamulatum* showing the similarity of UGT197 and Q8 regions between the two species and supporting the restriction/hybridization data from Gualtieri *et al.* (2006) for UGT197. Such similarity in banding patterns between the two species also has been shown by BACs from marker classes X18R and

TABLE 3

Contig analysis of ASGR-linked BAC clones in *P. squamulatum* and *C. ciliaris*

Marker	FPC contig no.[a]	BACs[b]											
Ugt197[c]	*1 and 16*	*C101*[d]	*C102*	*C103*	*C106*	*P201*	*P202*	*P203*	*P204*	*P205*	*P206*	*P207*	*P208*
	8	C100	C104	C105	C107	C110	C111						
	11	C108	C109	C112									
Q8M[c]	*2*	*C004*	*C005*	*C006*	*C010*	*C014*	*C015*	*C016*	*P102*	*P103*	*P106*	*P107*	
	3	*C001*	*C002*	*C008*	*C012*	*C013*	*C017*	*P101*					
	Singleton	P104	P105	P109	C018								
O7M[c]	5	P500	P503	P506	P507								
	7	C501	C518	C520									
	10	C509	C515	C522									
	Singleton	P508	P518										
W10M[c]	4	P900	P901	P902	P903								
X18R/W10M[c]	*15*	*P601*	*C901*										
	Singleton	P602											
U12H[c]	6	P800	P802	P803	P804								
	Singleton	P801											
R13[c]	9	P704	P705	P707									
	14	P706	P708	P709									
	Singleton	P701	P702	P703									
A14M[c]	13	P001	P002	P003	P004								
C4[c]	*12*	*C201*	*P400*										
	Singleton	C205											
A10H[c]	Singleton	P301	P303	C300									
Py503	17	P1001	P1002										
Py503	Singleton	P1000											
Px299	Singleton	P1100											
Px299	Singleton	C1200											
Pa265	Singleton	P1200											
Pq355	Singleton	P1300											
hhu27	Singleton	C1000											
P800 genes	Singleton	C1100											
P16R[c]	Singleton	C601	C602										
M02[c]	Singleton	C801	C802										
V4[c]	Singleton	C701											

[a] Each row represents one contig, except those marked "singleton" and 1 and 16, which were combined on the basis of FPC and RFLP data from Gualtieri *et al.* (2006). Italic data have BACs from both species forming one contig.

[b] BACs starting with "P" belong to the polyhaploid library while those starting with "C" belong to the buffelgrass library.

[c] SCAR markers are described in Ozias-Akins *et al.* (1998).

[d] Underlined BACs were used for FISH.

C4. To further test the degree of macrosynteny between the two species at the ASGR, orthologous BACs were used for FISH.

Macrosynteny of physically mapped BACs containing nonrecombining ASGR markers: To explore the degree of macrosynteny between *P. squamulatum* and *C. ciliaris* within the ASGR, we carried out multiple probe comparisons using FISH with ASGR-linked BAC clones (Table 3). BAC clones were considered orthologous if one or all of the following three criteria were satisfied: (1) BAC clones from the two species fell in the same contig during FPC analysis; (2) BAC clones from the two species shared one or more DNA sequences that matched the same GenBank sequence with BLASTx (*e*-value $<10^{-6}$) (Gualtieri *et al.* 2006; J. A. Conner, S. Goel, G. Gunawan, M.-M. Cordonnier-Pratt, C. Liang, L. Pratt and P. Ozias-Akins, unpublished results); (3) sequences from one BAC were present on the other as shown by PCR/hybridization (Gualtieri 2006; J. A. Conner, S. Goel, G. Gunawan, M.-M. Cordonnier-Pratt, C. Liang, L. Pratt and P. Ozias-Akins, unpublished results). Some regions within a pair of orthologous BACs would not have necessarily been represented in both BACs, such as a BAC end or nongenic regions. ASGR-linked BAC clones were physically mapped on metaphase and pachytene chromosome spreads of *P. squamulatum* and *C. ciliaris* using P208 as an anchor marker for the ASGR. P208 was chosen as an anchor since it produced a clean bright signal in both species, probably due to the tandem duplication, and has been very well characterized for its localization on both species (Goel *et al.* 2003; Akiyama *et al.* 2004, 2005).

Most of the BAC clones tested showed syntenic positions between the two species, although an inverted

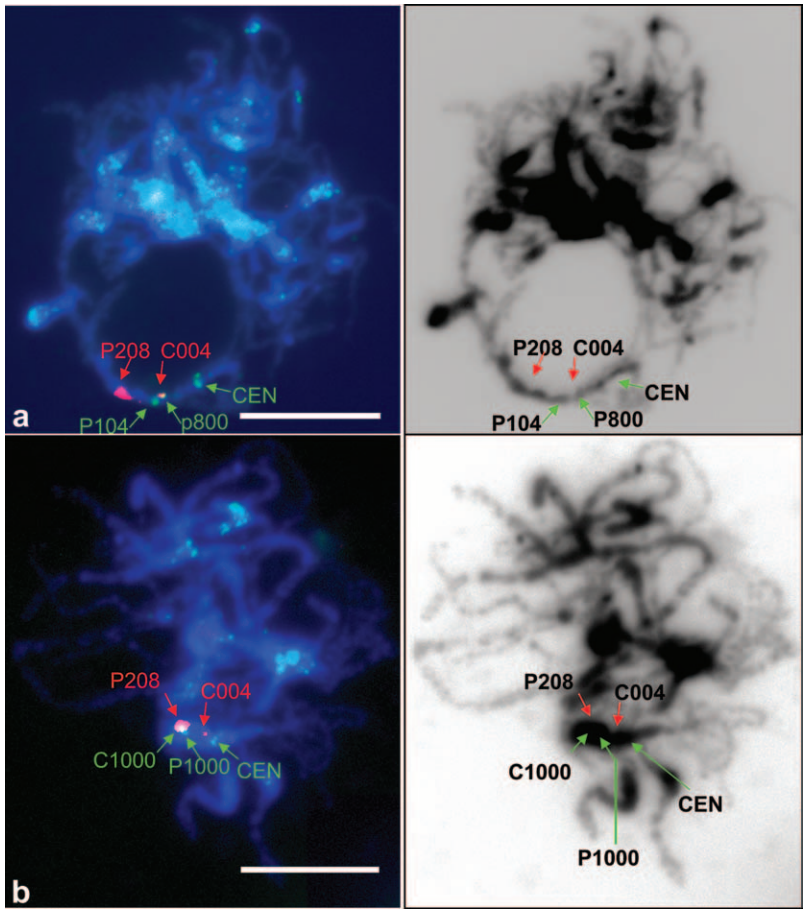

FIGURE 1.—Physical localization of multiple BAC clones on pachytene chromosomes of *C. ciliaris*. A centromeric BAC (CEN) and the marker UGT197-containing BAC (P208) were used to orient the order of signals. The heterochromatic nature of the ASGR can be better visualized in the inverted images. Bars, 10 μm.

order, as reported earlier (GOEL *et al.* 2003; AKIYAMA *et al.* 2004), was confirmed and extended (Figures 1 and 2). Some of the BAC clones (P800, P1100 and their *C. ciliaris* orthologs C1100 and C1200) (Table 3) produced a signal in two large blocks flanking a low-copy region of the ASGR in *P. squamulatum* due to the presence of repetitive DNA as previously reported (AKIYAMA *et al.* 2004). In *C. ciliaris* these BAC clones produced a signal that was dispersed over the whole genome. To eliminate repetitive DNA from the BAC probe, a shotgun library of subclones from P800 (average insert size of ~2 kb) was screened using labeled B-12-9 total genomic DNA. A subset of subclones that did not hybridize with total genomic DNA, and thus were less likely to contain repetitive DNA, were pooled together and labeled for FISH (~20 kb). This DNA pool produced a single discrete signal in *P. squamulatum* (Figure 2a) and could give a detectable signal on the ASGR in *C. ciliaris* (Figure 1a) although minor signals inconsistently appeared on other chromosomes. P1100 produced a clean signal in *P. squamulatum* when unlableled P602 DNA was used as a block to suppress the signal produced due to common repetitive DNA (Figure 2a). In *C. ciliaris*, however, P1100 produced a signal dispersed over the entire genome, which persisted even after using P602 as a block. Orthologous BACs for P800 and P1100 isolated from the *C. ciliaris* library (Table 3) also contained high-copy sequences and could not produce a clear signal in *C. ciliaris*.

Two sets of orthologous BAC clones (C101 and P208; C004 and P102) were used to determine the corresponding physical locations in *C. ciliaris*. Dual-labeled FISH with these pairs of BACs produced overlapping signals (data not shown), confirming their orthologous relationship. Pairs of BAC clones that did not contig together but belonged to the same marker class (C001 and C004; P102 and P104; C108 and P208) always produced overlapping or adjacent signals on pachytene chromosomes of *C. ciliaris*.

The order of multiple BAC clones was confirmed on pachytene spreads of *C. ciliaris* by dual-labeling of pairs of putatively alternating clones (Figure 1, a and b). These BAC clones were oriented with the help of centromere detection using a centromeric BAC isolated from the *C. ciliaris* library as previously described (GOEL *et al.* 2003). The order of BAC clones was decided after examining a minimum of 8–10 different spreads. After determining the putative order of pairs of BAC clones, more than two BAC clones were localized together to enable visualization of their comparative order on the chromosome. The comparative order of the ASGR-linked BAC clones in *P. squamulatum* and *C. ciliaris*, as determined from multiple experiments, is illustrated in Figure 3.

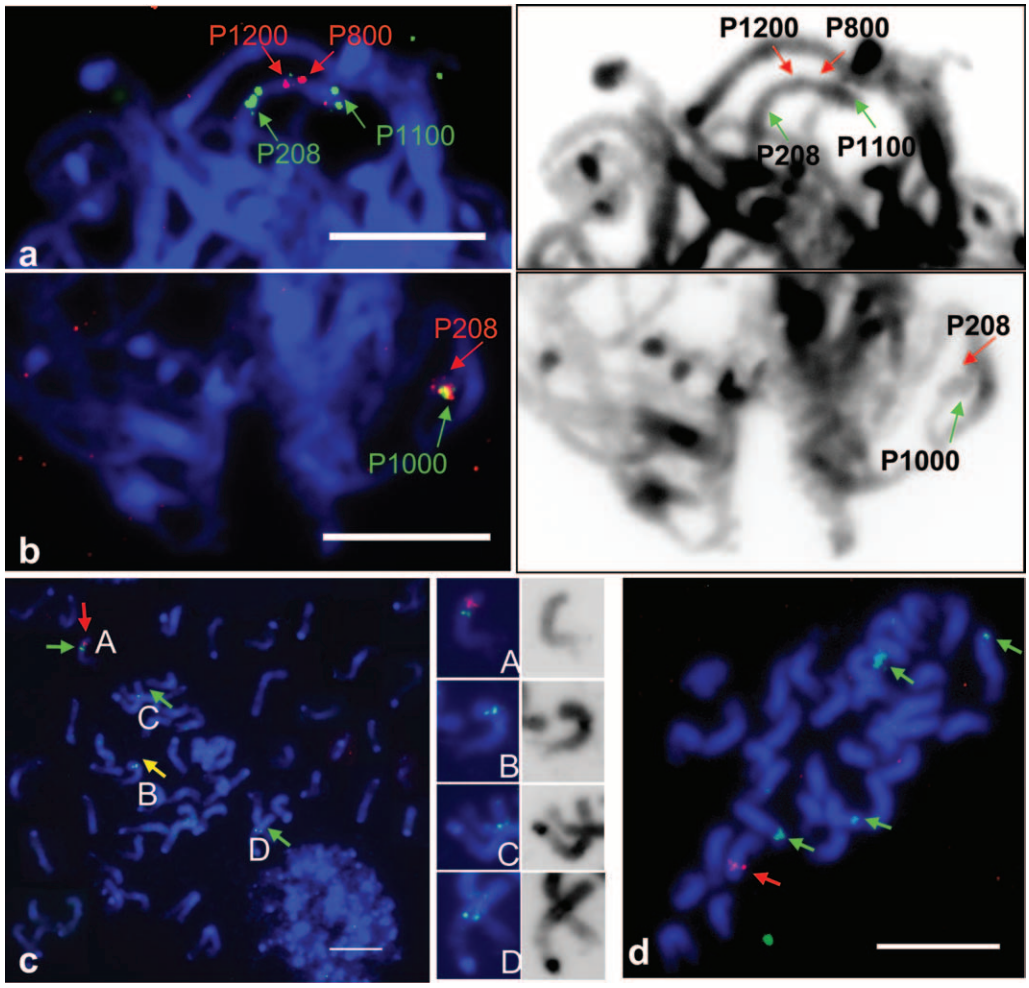

FIGURE 2.—Physical mapping of BAC clones containing AFLP markers. (a and b) Pachytene chromosomes in *P. squamulatum*. P208 was used as an anchor marker. (c and d) Localization of BAC P1300 containing the marker that recombines with the ASGR. (c) Metaphase chromosomes of *P. squamulatum*. Red arrow indicates P208 signal (red), green arrows indicate P1300 signals (green), and yellow arrow indicates the putative homolog of the ASGR-carrier chromosome. (d) Localization of P1300 on *C. ciliaris* metaphase chromosomes. Red arrow indicates P208 signal (red); green arrows indicate the P1300 signal (green). P1300 does not localize to the ASGR-carrier chromosome identified by P208. (A–D insets) The enlarged chromosomes with signal, marked by corresponding letters. Note the morphological similarity between A and B.

In *P. squamulatum*, BACs P1000 (marker py503), P1100 (marker px299), and P1200 (marker pa265) showed clearly separated signals within the ASGR (Figure 2, a and b). Of these three, P1100 was the only BAC that required blocking of repetitive DNA sequences with BAC P602, a clone that contains a partially characterized Opie-2-like retroelement (AKIYAMA *et al.* 2004), to produce a clear discrete signal. BAC P1100 was the most distal in its position and located close to the telomere of the short arm of the ASGR-carrier chromosome while BACs P1000 and P1200 showed signal on the centromeric side of P1100. In *C. ciliaris*, among the three BACs containing AFLP markers from *P. squamulatum*, only one (P1000) could be localized on the ASGR-carrier chromosome (Figure 1b). BAC P1100 did not show clear signal due to hybridization with repetitive sequences on other chromosomes while P1200 never produced a detectable signal in *C. ciliaris*.

Among all the BACs used for FISH, P1200 (marker px299) and P301 (marker A10H) did not produce any signal in *C. ciliaris*, even after repeated attempts. P303, which contigs with P301 at a stringency of 10^{-10} but not of 10^{-12}, produced a strong signal over the entire *C. ciliaris* genome, which probably was due to repetitive sequences in the BAC clone. Because of this distributed signal, we could not confirm the absence/presence of P303 in the ASGR of *C. ciliaris*. In the ASGR of *P. squamulatum*, P1200 and P301 are physically adjacent to one another (Figure 2). Using shotgun sequence information from BAC P1200, primer pairs were designed from those regions of the BAC that did not return any matching sequence from a BLASTx query. One of these primer pairs (Table 1) could differentiate apomictic *P. squamulatum* and BC7 line 58 (GOEL *et al.* 2003) from the sexual species *P. glaucum*, although the same primer pair could not amplify any sequence from *C. ciliaris* from either apomictic or sexual genotypes. The ~800-bp amplicon from BAC P1200 hybridized to nine BACs from the *C. ciliaris* BAC library, all of which produced a similar band profile after *Hin*dIII digestion and probing with the radiolabeled ~800-bp amplicon, suggesting that they originated from the same/similar locus (data not shown). FISH with two of these BAC clones in *C. ciliaris* produced background noise on chromosomes due to repetitive sequences but no signals were localized on the ASGR-carrier chromosome, suggesting that the region containing P1200 sequences may be missing from the ASGR in *C. ciliaris*. On the contrary, marker A14M previously had been shown to be absent from *C. ciliaris* by both PCR and hybridization (ROCHE *et al.*

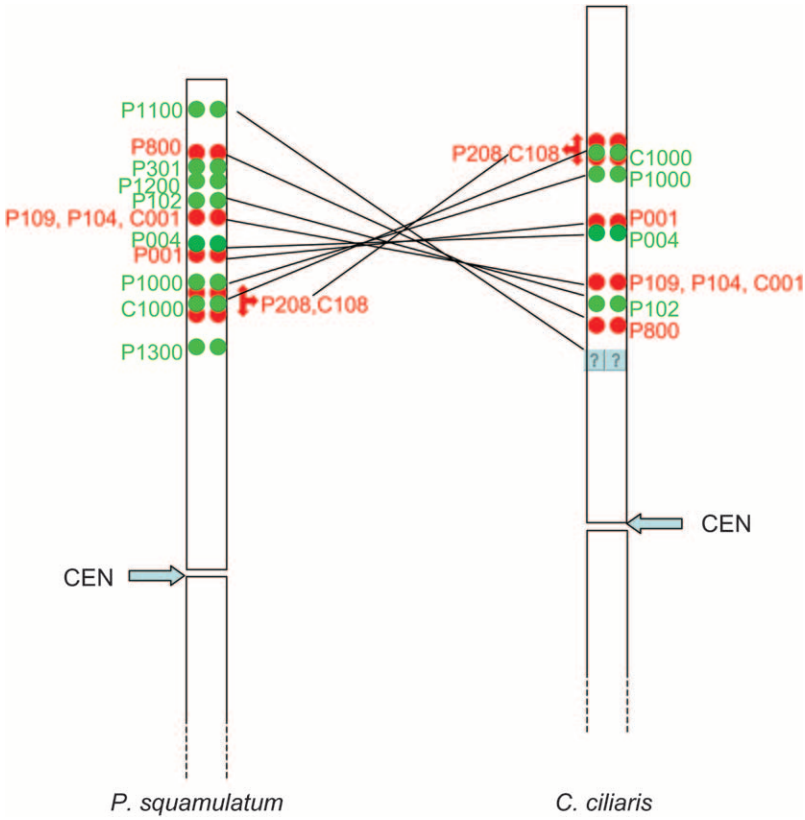

FIGURE 3.—Diagrammatic of the comparative order of different BAC clones in the ASGRs of *P. squamulatum* and *C. ciliaris* based on data from fluorescence *in situ* hybridization. Figure is not to scale and represents approximate relative positions of FISH signals. BACs beginning with "P" were from *P. squamulatum*; those beginning with "C" were from *C. ciliaris*. Marker and contig assignment can be found in Table 3.

1999), but in this investigation a BAC containing this marker (P001) could be localized near BAC P208, which is syntenic to its location in *P. squamulatum*. A small deletion could account for the difference in marker detection *vs.* whole-BAC detection.

Physical mapping of BACs containing ASGR recombinant markers: P1300, the only BAC containing a recombining AFLP marker (pq355) from *P. squamulatum*, showed signal on four chromosomes in *P. squamulatum*, one of which was the ASGR-carrier chromosome (Figure 2c). This discrete signal was most proximal to the centromere of any of the other BACs analyzed by FISH. There were weak signals on some other chromosomes, probably due to repetitive sequences present on BAC P1300, but these signals were not reproducible. One pair of the four hybridizing chromosomes had a heterochromatic region at one of the telomeric ends and easily could be differentiated from the ASGR-carrier chromosome (Figure 2c, insets C and D). One chromosome of the pair was much more consistent than the other in showing a discrete P1300 signal. The fourth chromosome showing P1300 signal had features similar to the ASGR-carrier chromosome except for a smaller short arm (Figure 2c). The longer short arm of the ASGR-carrier chromosome compared with its putative homolog may have resulted from an insertion/translocation event. A statistical comparison (*t*-test) of the short arms of the ASGR-carrier chromosome and the putative homologous chromosome in seven different spreads showed a significant difference ($t = 2.31$, $P = 0.038$) in length while no significant difference was found for long arm length ($t = 0.857$, $P = 0.407$). Surprisingly, when BAC P1300 was hybridized to *C. ciliaris*, four chromosomes showed signal, but none was the ASGR-carrier chromosome (Figure 2d), suggesting that a translocation may have contributed to the current heteromorphic structure of the ASGR-carrier chromosome in *P. squamulatum*.

Since the AFLP map of *P. squamulatum* provided only one closely recombining marker, we chose a second recombining marker from buffelgrass (JESSUP *et al.* 2002), linked with the ASGR at a distance of 10.7 cM, to investigate by using BAC–FISH. The probe used to generate this marker in buffelgrass was from a sorghum cDNA library (HHU27, GenBank nos. H54993 and H54994); however, we isolated our probe from the rice BAC (OSJNBa0067K08, AL606627; FENG *et al.* 2002) that had the greatest similarity to HHU27 (86% by BLASTn). A fragment (873 bp) was amplified using primers (Table 1) complementary to segments conserved between the two species and targeting the 5′ portion of the first exon from HHU27. Three *C. ciliaris* BACs containing this amplicon were used for FISH, but only one of the three BAC clones (C1000) produced a single discrete signal on the ASGR-carrier chromosome in both species. Similar to other BACs that contained AFLP and SCAR markers, this cDNA marker-containing BAC also showed a hemizygous hybridization pattern. On pachytene chromosomes of *C. ciliaris*, this BAC produced an overlapping signal with P208 (marker

UGT197) and was sometimes observed to be positioned between the two signals and large signal produced by P208 (Figure 1b). In our previous mapping study, marker UGT197 did not show any recombination with apospory; therefore, we would not expect a marker that was positioned between two copies of UGT197 to show recombination. To test whether BAC C1000 contained any marker that could be shown to segregate with apospory in our *C. ciliaris* mapping population, we designed three primer pairs (Table 1) on the basis of the shotgun sequence information generated from this BAC (J. A. Conner, S. Goel, G. Gunawan, M.-M. Cordonnier-Pratt, C. Liang, L. Pratt and P. Ozias-Akins, unpublished results). The sequences used for designing the primers were from those shotgun subclones that did not match any known sequences by BLASTx to GenBank. All three primer pairs could differentiate between the sexual and the ASGR-carrier plants obtained from a cross between sexual and apomictic *C. ciliaris* and amplified as dominant markers in apomicts (data not shown). These three dominant markers did not show any recombination with apospory in the *C. ciliaris* population of 61 apomicts and 24 sexuals.

DISCUSSION

Recombination in the ASGR-carrier chromosome: Previous mapping studies from our lab have not been able to detect any recombination at or near the ASGR in *P. squamulatum*. The only recombinant marker reported up to now, UGT204, is at a considerable genetic distance from the ASGR (Ozias-Akins *et al.* 1998). The lack of recombination in the ASGR may be due to a combination of factors such as its heterochromatic nature, an asynaptic or desynaptic short arm due to the hemizygous nature of the ASGR and a heterozygous inversion in *P. squamulatum*, and hemizygosity plus location near a centromere in *C. ciliaris* (Ozias-Akins *et al.* 1998; Grossniklaus *et al.* 2001; Goel *et al.* 2003; Akiyama *et al.* 2004). An AFLP marker generated in this study has, for the first time, detected recombination near the ASGR in *P. squamulatum*. Localization of the BAC clones containing AFLP markers by FISH has given further insight into the recombination behavior of the short arm of the ASGR-carrier chromosome. P1300, which carries the only recombining marker, Pq355, localized most proximal to the centromere in *P. squamulatum*, as would be expected if the distal portion of the ASGR-carrier chromosome were nonrecombinant. Furthermore, BAC P1300 is the only BAC that has not shown a hemizygous hybridization pattern since it hybridized not only with the ASGR-carrier chromosome but also with its apparent homolog. Comparison of the morphological features of the ASGR-carrier chromosome and its homolog provides evidence that the ASGR could be an insertion/translocation on the short arm of the ASGR-carrier chromosome of *P. squamulatum*. Pairing behavior of the ASGR-carrier chromosome further supports this conclusion in that the short arm of this chromosome does not synapse in at least 80% of the spreads during male meiosis although the long arm clearly is paired with one other chromosome (Akiyama *et al.* 2004). In contrast to *P. squamulatum*, the ASGR is present on one of the four chromosomes with rDNA loci in *C. ciliaris* (Goel *et al.* 2003), and evidence for heteromorphism from ideograms containing these four chromosomes suggests that the ASGR may be an insertion on one of them (Akiyama *et al.* 2005). The BAC (P1300) containing the recombining marker did not localize to the ASGR-carrier chromosome in *C. ciliaris*, indicating a disruption of synteny between the two species outside of the ASGR, which argues that the ASGR can change location in the genome while being maintained as a haplotype. The potential for recombination in a genome that contains two ASGR chromosomes, provided they are polymorphic, could be useful for map-based cloning of the apospory-governing genes. Our survey of 16 Pennisetum and two Cenchrus species has identified one that shows the presence of two ASGR-carrier chromosomes (Y. Akiyama, S. Goel, W. W. Hanna, P. Ozias-Akins, unpublished results).

Jessup *et al.* (2002) reported recombination of numerous markers and apospory in *C. ciliaris* even though some markers previously had been shown to be tightly linked with apospory (UGT197 and Q8) in both *P. squamulatum* (Ozias-Akins *et al.* 1998) and a different population of *C. ciliaris* (Roche *et al.* 1999). We investigated one of their recombining markers (HHU27) by isolating a BAC clone from *C. ciliaris* (C1000) that contained sequences from this marker. According to the map published by Jessup *et al.* (2002), two of these markers (UGT197 and HHU27) flanked the apospory locus on opposite sides at a distance of 1.4 and 10.7 cM, respectively. In our analysis we could not detect any recombination between SCAR markers developed from BAC C1000 and the apospory trait. Population differences, marker/probe differences, or phenotyping errors could account for this discrepancy between the two studies. Although the two markers were separated by a 12-cM genetic distance in Jessup *et al.* (2002) and flanked the apospory locus, the physical distance on pachytene chromosomes of *C. ciliaris* is relatively small between BAC C1000 and the UGT197-containing BAC P208 (in fact, the C1000 signal often falls between two P208 signals). If there are no phenotyping (marker or trait) errors, the gene(s) controlling apospory would be located in a relatively small portion of the ASGR. An alternative explanation that could account for the small genetic distance between UGT197 and apospory is that the cluster of markers that is shown as recombining with apomixis in Jessup *et al.* (2002) actually should include apospory in the cluster. This separation of the UGT197 cluster from apospory was based on the phenotype of a single sexual individual. It would be interesting to examine progeny of this sexual

individual to determine whether an epistatic "suppressor" of apomixis, proposed by Ozias-Akins *et al.* (2003) to explain the earlier segregation data of Taliaferro and Bashaw (1966), might be present. Such a map revision, however, still would not reconcile the two results with HHU27.

Synteny between *P. squamulatum* and *C. ciliaris* at the ASGR: Similarity between the ASGR of *C. ciliaris* and *P. squamulatum* already has been shown by shared SCAR markers where 10 of the 12 markers reported in *P. squamulatum* were present in *C. ciliaris* (Roche *et al.* 1999). Our present observations provide evidence for extensive macrosynteny between the ASGR of the two species but also emphasize some subtle differences that may reflect local disruptions of microsynteny. Disruption of microsynteny due to small insertions or deletions may be the explanation for the absence of marker A14M from *C. ciliaris* as detected by PCR and Southern hybridization (Roche *et al.* 1999), while the hybridization of the BAC containing this marker (P001) is syntenic in *C. ciliaris* with its position in *P. squamulatum*. Conversely, the marker A10H that is present in *C. ciliaris* (Roche *et al.* 1999) did not retrieve a BAC that could be localized in *C. ciliaris*. BAC P1200, which localized adjacent to an A10H-containing BAC in *P. squamulatum*, also could not produce any detectable signal in *C. ciliaris*. These observations could indicate that a large insertion/deletion is present in this region. Lack of synteny between the two species in this part of the ASGR suggests that this region is not required to confer the aposporous trait.

BAC clones P1100, P800, and their *C. ciliaris* orthologs, which produced signal dispersed over the entire genome of *C. ciliaris*, provided further evidence for the tendency toward a larger number of copies of ASGR-related repetitive sequences in *C. ciliaris* than in *P. squamulatum* (Roche *et al.* 1999). Similarly, BAC P602 hybridizes to the distal half of the short arm of the ASGR-carrier chromosome in *P. squamulatum* but hybridizes across the genome in *C. ciliaris* (Akiyama *et al.* 2005). These results implicate a *C. ciliaris*-like ancestor as either a direct or indirect donor of the ASGR to *P. squamulatum*. The alternative hypothesis that the ASGR originated in *P. squamulatum* and that repeat elements proliferated throughout the genome of *C. ciliaris* subsequent to its movement is considered unlikely.

Numerous comparisons of genome structure between closely related species have been carried out where comparative mapping generally has revealed colinear segments (Schmidt 2002). Although gene order can be highly conserved, intergenic sequences are variable, particularly in larger genomes like maize where such regions consist largely of nested retrotransposons (SanMiguel *et al.* 1996; Chen *et al.* 1998; Tikhonov *et al.* 1999; Paterson *et al.* 2000). The two species that we studied, *C. ciliaris* and *P. squamulatum*, are related to members of a paraphyletic Pennisetum in which a monophyletic Cenchrus clade is embedded (Doust and Kellogg 2002). *C. ciliaris* has been placed in the genus Pennisetum in earlier taxonomic treatments (Jauhar 1981). The two species are not sexually compatible, although a single F_1 hybrid between diploid pearl millet and tetraploid *C. ciliaris* has been produced but was sterile (Read and Bashaw 1974). Considering the close relationship of these two apomictic species and the fact that the ASGR governs apomixis in both species, a highly syntenic relationship is expected at the ASGR. However, it is still possible that repetitive sequences and retrotransposons at the ASGR (Akiyama *et al.* 2004; J. A. Conner, S. Goel, G. Gunawan, M.-M. Cordonnier-Pratt, C. Liang, L. Pratt and P. Ozias-Akins, unpublished results) could disrupt this synteny at places and could increase the comparative intergenic distances.

Microsynteny between *P. squamulatum* and *C. ciliaris* at the ASGR is supported by contig analysis where ASGR-linked BACs isolated from *C. ciliaris* and *P. squamulatum* often share restriction patterns and common hybridizing fragments (Table 3; Gualtieri *et al.* 2006). Within a species, it is possible that two contigs were merged by FPC due to highly similar restriction fragment patterns but actually may be nearly identical duplications. This type of merge occurred between clones from two different species, which clearly do not form a true contig but have extremely high similarity, even at the DNA sequence level where nearly identical genes have been identified on orthologous BAC clones (J. A. Conner, S. Goel, G. Gunawan, M.-M. Cordonnier-Pratt, C. Liang, L. Pratt and P. Ozias-Akins, unpublished results). For example, P208 and C101 contain orthologous sequences as shown by sample sequencing (Gualtieri *et al.* 2006; J. A. Conner, S. Goel, G. Gunawan, M.-M. Cordonnier-Pratt, C. Liang, L. Pratt and P. Ozias-Akins, unpublished results), restriction fragment hybridization patterns (Gualtieri *et al.* 2006), assembly into the same FPC contig, and detection of duplicated FISH signals in both species. None of the other BACs used in this study have shown duplication of FISH signals. FISH, where different BAC contigs belonging to the same marker class produced overlapping/adjacent FISH signals, suggests that incomplete coalescence of contigs was most likely due to the low genome coverage of the BAC libraries (Roche *et al.* 2002).

Conclusions: This investigation provides a comprehensive comparative view of the structure of the ASGR between apomictic genotypes from two closely related species. The genetic mapping study confirms a lack of recombination at the ASGR in *P. squamulatum* and further supports the hemizygous nature of the ASGR as a major factor preventing recombination even though the ASGR in *P. squamulatum* also is inverted. Since the ASGR in *C. ciliaris* displays the same hemizygous nature, it is unlikely that further recombination studies in *C. ciliaris* will provide the ability to fine map the ASGR due to its large size in both species (Akiyama *et al.* 2004, 2005). In both *P. squamulatum* and *C. ciliaris*, the ASGR

appears to be an insertion/translocation on the ASGR-carrier chromosomes when compared to their putative homologs (Akiyama *et al.* 2005; this study). On the basis of these observations, we speculate that the present position of the ASGR in *P. squamulatum* is the consequence of chromosomal rearrangements (inversion and translocation) as well as introgression of the rearranged chromosome by hybridization during its divergence from *C. ciliaris* or a common ancestor and that macrosynteny outside of the ASGR might not be expected on the ASGR-carrier chromosome. Such chromosomal rearrangements could be better tolerated in asexual *vs.* sexual species since the preservation of meiotic function is not as critical. Gross chromosomal rearrangements also have been shown to cause amplification of genomic fragments (Infante *et al.* 2003) and could have contributed to the complexity of the ASGR in some species. Nevertheless, within the ASGR, synteny (marker/BAC content and order) is remarkably conserved between *C. ciliaris* and *P. squamulatum*, even among hemizygous regions, suggesting that a large part of the sequence divergence in the ASGR compared with putative homologs preceded gross chromosomal rearrangements. Where synteny is interrupted between two apomicts, genes essential for apomixis are not expected to be present. Nevertheless, the size of the ASGR will continue to be a limiting factor in map-based cloning of gene(s) required for conferring the trait of apospory unless it can be reduced by further interspecies comparisons, identification of recombinant genotypes, or deletion mutagenesis. Studying the inheritance of the ASGR in a genotype carrying two ASGR chromosomes, provided they are polymorphic, might provide a greater chance to reduce the effective size of the ASGR through recombination.

Recent studies of apomixis have emphasized the involvement of common elements between apomixis and sexual reproduction (Tucker *et al.* 2003). Considering the residual sexuality among obligate apomicts and overlap in reproductive events for apomixis and sexual reproduction (Grimanelli *et al.* 2001; Tucker *et al.* 2003), it might be difficult to identify gene(s) responsible for the apomictic trait solely through gene expression without also analyzing the complex locus governing the trait. As suggested by the present results, if the ASGR is being maintained as a haplotype, there should be forces to select against disruption of the region, although recent origin could be another explanation. These questions might be answered by analyzing the ASGR in other related species of the Pennisetum/Cenchrus complex.

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