Theor Appl Genet (2005) 111: 1042–1051
DOI 10.1007/s00122-005-0020-5

ORIGINAL PAPER

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High-resolution physical mapping reveals that the apospory-specific genomic region (ASGR) in *Cenchrus ciliaris* is located on a heterochromatic and hemizygous region of a single chromosome

Received: 6 January 2005 / Accepted: 9 June 2005 / Published online: 17 August 2005
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Abstract An apomictic mode of reproduction known as apospory is displayed by most buffelgrass *(Cenchrus ciliaris)* genotypes, but rare sexual individuals have been identified. Previously, intraspecific crosses between sexual and aposporous genotypes allowed linkage to be discovered between the aposporous mode of reproduction and nine molecular markers that had been isolated from an aposporous relative, *Pennisetum squamulatum*. This region was described as the apospory-specific genomic region (ASGR). We now show an ideogram of the chromosome complement for aposporous tetraploid buffelgrass accession B-12-9 including the ASGR-carrier chromosome. The ASGR-carrier chromosome has a region of hemizygosity, as determined by in situ hybridization of BAC clones and unique morphological characteristics when compared with other chromosomes in the genome. In spite of its unique morphology, the ASGR-carrier chromosome could be identified as one of the chromosomes of a meiosis I quadrivalent. A similar partially hemizygous segment was also detected in the ASGR-carrier chromosome of the aposporous buffelgrass genotype, Higgins, but not in the sexual accession B-2S. Two non-recombining BACs linked to apospory were physically mapped on a highly condensed chromatin region of the short arm of B-12-9, and the distance between the BACs was estimated to be ~11 Mbp, a distance similar to what previously has been shown in *P. squamulatum*. The short arm of the ASGR-carrier chromosome was highly condensed at pachytene and extended only 1.7–2.7 fold that of mitotic chromosomes. Low recombination in the ASGR may partially be due to its localization in heterochromatin.

Communicated by J.S. Heslop-Harrison

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Introduction

Apomixis is a fascinating trait in plants because it allows a plant to avoid sexual reproduction in favor of a maternal derivation of seeds (Grimanelli et al. 2001; Koltunow 1993; Nogler 1984; Ozias-Akins et al. 2003). The application of apomixis to plant breeding could have tremendous utility because it provides a mechanism for hybrid progeny to circumvent segregation and fix heterosis. Unfortunately, the trait has not been found in major commercial crops and is rare in wild crop relatives; therefore, attempts to transfer apomixis into major crops through traditional breeding methods have not been very successful (Dujardin and Hanna 1989). Apospory is only one form of apomixis wherein embryo sacs arise from somatic cells of the nucellus or integuments. Apospory is speculated to be controlled by a single locus or group of tightly clustered loci in *Cenchrus ciliaris* L. (Jessup et al. 2002; Roche et al. 1999), *Pennisetum squamulatum* (Ozias-Akins et al. 1998), and *Paspalum simplex* (Labombarda et al. 2002).

Buffelgrass (*C. ciliaris* syn. *P. ciliare* L. Link), an aposporous apomict, has been used to study phenotypic expression of the trait, its inheritance, its linkage with molecular markers and for comparisons at the genomic level with related species (see Ozias-Akins et al. 2003 for review). Although the chromosome number in *C. ciliaris* can vary from 18 to 56 (Visser et al. 1998a), most genotypes are tetraploid (2n = 4x = 36). The genome size of the tetraploid is ~1500 Mbp/C (Roche et al. 2002), which is about four times the size of the rice genome

(~400 Mbp/C; Arumuganathan and Earle 1991; Ohmido et al. 2000).

Even though buffelgrass is predominantly apomicitc, sexual accessions have been found which made intraspecific crosses possible (Bashaw 1962). Crossing a sexual maternal parent with an aposporous paternal parent has led to theories of two-gene, apospory-hypostatic (Bashaw 1962) and single-gene, apospory-dominant inheritance (Sherwood et al. 1994). Based on segregation ratio differences when two aposporous male parents were used in crosses, Sherwood et al. (1994) proposed that the tetraploid genotypes B-12-9 and Higgins had allelic compositions of Aaaa and AAaa, respectively. Sexual tetraploid genotype B-2S would be designated as aaaa.

Ozias-Akins et al. (1998) found 12 molecular markers linked to apospory in *P. squamulatum* where they were assayed as sequence-characterized amplified regions (SCARs). None of the 12 SCAR markers recombined in *P. squamulatum*. Of the six SCARs that also could be specifically amplified in apomictic *C. ciliaris*, only one recombinant individual for one marker (X18) was identified (Roche et al. 1999). Three other SCARs were mapped as RFLPs, but no recombination was observed. Therefore, the evidence supported conservation of sequence between the apospory-specific genomic regions (ASGR) of *P. squamulatum* and *C. ciliaris*. To further explore the degree of conservation between the two species, bacterial artificial chromosome (BAC) libraries were constructed and used to isolate large-insert clones containing the apomixis-linked markers (Roche et al. 2002). Fluorescence in situ hybridization (FISH) on mitotic chromosomes with the BACs that contained primarily low-copy sequences demonstrated that the ASGR in *C. ciliaris* and *P. squamulatum* was hemizygous (Goel et al. 2003). The FISH results showed that the ASGR was positioned near the telomere in *P. squamulatum*, while in *C. ciliaris* it was located close to the centromere of a chromosome that also contained the 18S rDNA locus. Eleven ASGR-linked, low- and high-copy BACs (as determined by hybridization of BACs with labeled total genomic DNA) were microscopically positioned onto pachytene complements of *P. squamulatum* (Akiyama et al. 2004). This high-resolution FISH revealed that the high-copy BACs were distributed in two hemizygous blocks on the ASGR-carrier chromosome and that the low-copy BACs were located between the blocks. The entire hemizygous region in *P. squamulatum* was estimated to be approximately 50 Mbp. Repetitive elements in the high-copy BACs were sequenced and shown to have similarity with the Opie-2 retrotransposon family from maize (Akiyama et al. 2004).

Although the ASGR-carrier chromosome in *P. squamulatum* was shown to pair with a presumed homolog, the short arm that bears the hemizygous ASGR was largely asynaptic or desynaptic (Akiyama et al. 2004); therefore, recombination would be rare or absent. The ASGR in buffelgrass was also observed to be largely non-recombinant (Roche et al. 1999), although conflicting results have been published by Jessup et al. (2002) who found a cluster of markers that mapped 1.4 cM from apospory but no marker that co-segregated with the trait. Experimental data from a diplosporous apomict, *Tripsacum dactyloides*, also indicated repressed recombination among markers linked with apomixis compared with the syntenic region in sexual, diploid *Tripsacum* or in maize (Grimanelli et al. 1998).

In the present study, we have investigated the association of the ASGR-carrier chromosome with other chromosomes at meiosis and have constructed a quantitative ideogram of aposporous tetraploid *C. ciliaris* B-12-9 by using image analysis to document heteromorphism of the ASGR-carrier chromosome. In addition, we investigated the distribution in buffelgrass of the Opie-2-like retroelement found to be flanking the low-copy region of the ASGR in *P. squamulatum*.

Materials and methods

Plant materials

Two aposporous accessions, B-12-9 and Higgins, and one sexual accession, B-2S, were used as plant materials. Each of these lines had been received in 1996 from D.L. Gustine, USDA-ARS, University Park, PA, USA, have been maintained since that time by vegetative propagation and were parents of the mapping populations used in Gustine et al. (1997) and Roche et al. (1999). For meiotic chromosome preparation, immature panicles of B-12-9 cultured in the field were collected in summer and fixed in 3 ethanol:1 acetic acid (v/v). For mitotic chromosome preparation, the root tips were collected from plants in the greenhouse and soaked in a saturated solution of α-bromonaphthalene at 4°C for 3 h prior to fixation.

BACs

The construction of BAC libraries and isolation of ASGR-linked BAC clones (C001, C013 and P109 derived by SCAR Q8M; C101 and, P208 – SCAR ugt197) have been reported (Roche et al. 2002). BACs containing SCAR markers with repetitive DNA (P602 – SCAR X18R) were isolated by PCR screening of pooled BAC DNA (Liu et al. 2000; Akiyama et al. 2004). The BACs named Cxxx and Pxxx were derived from *C. ciliaris* B-12-9 and the polyhaploid apomictic line MS228-20, respectively.

Chromosome preparation and fluorescent in situ hybridization (FISH)

Cytological experiments were carried out according to Akiyama et al. (2004). For mitotic chromosome preparation, the root tips were treated by an enzyme mixture (1.33% Cellulase Onozuka RS, 0.5% Macerozyme R-200, 0.23% Pectolyase Y-23, 0.33 mM EDTA, pH = 4.2

(Fukui 1996) at 37°C for 80 min. For meiotic chromosome preparation, approximately 1.0 mm anthers were selected and treated with an enzyme mixture (0.3% Cellulase RS, 0.3% Pectolyase Y23, 0.3% cytohelicase, 30 mM citrate buffer, pH = 4.5) (Zhong et al. 1996) at 37°C for 7 h. After enzyme treatment, cells were spread on glass slides.

For FISH, the slides were pretreated in 0.005% Pepsin/0.01 N HCl at 37°C for 90 s, in 1 × phosphate-buffered saline (PBS) for 5 min, in 1% formaldehyde (buffered with 1 × PBS + 50 mM $MgCl_2$), 1 × PBS for 5 min, and dehydrated sequentially in 70, 95, and 100% ethanol at room temperature for 5 min each. Slides were air dried then denatured at 85°C with 70% formamide in 2 × SSC for 80 or 180 s for mitotic and meiotic chromosome spreads, respectively. Spreads were incubated with denatured hybridization mixture containing approximately 1 ng/μl biotin- or digoxigenin-labeled probe, 5% dextran sulfate (M = 500,000), 50% formamide, 2 × SSC in a humidified chamber at 37°C for at least 12 h. The digoxigenin-labeled probes were detected with fluorescein using a kit (Roche, Indianapolis, IN, USA). Biotin-labeled probes were detected with texas-red streptavidin (Vector Laboratories, CA, USA) and biotinylated anti-streptavidin (Vector Laboratories) for a second layer of texas-red streptavidin. After detection, the slides were mounted in Vectashield (Vector Laboratories) containing 1.5 μg/ml DAPI and observed under a fluorescence microscope, Olympus BX50.

Image analysis

The images of chromosomes were captured by Sensys CCD camera (Photometrics, AZ, USA) and Image Pro ver 4.1 software (Media Cybernetics, IA, USA). The quantitative ideogram for B-12-9 was constructed by using Object-Image 2.10 (http://simon.bio.uva.nl/object-image.html) with CHIAS3 (Kato and Fukui 1998) on a Macintosh Power Book.

Results

Construction of a quantitative ideogram for *C. ciliaris* B-12-9

The chromosomes of *C. ciliaris* are difficult to distinguish from one another because of their similar morphology and size. Therefore, FISH with 5S rDNA, 18S rDNA, and a BAC clone containing an apomixis-linked marker (BAC C101 containing SCAR ugt197) was used for identification of the chromosomes after characterization of DAPI-stained spreads (Fig. 1a, b). Digoxigenin-labeled 18S rDNA and biotin-labeled BAC DNA initially were applied, followed by a second probing with digoxigenin-labeled 5S rDNA. Signals from probes for 18S rDNA were detected on the distal end of the short arm of four chromosomes. BAC clone C101 also was detected on the short arm of the 18S rDNA-bearing chromosome as previously reported (Goel et al. 2003). The signal of C101, which represented one out of two ugt197-containing contigs from buffelgrass (Roche et al. 2002) was sometimes resolved as two hybridizing sites in close proximity. Very small signals from C101 also were detected at the centromere of the ASGR-carrier chromosome and all other chromosomes (Fig. 1a). Two types of chromosomes with 18S rDNA loci were observed, namely three small chromosomes and a mid-sized chromosome that also hybridized with C101 (Fig. 1a,b). The small 18S rDNA-bearing chromosomes were presumed to be homologs and were numbered chromosome 8 according to chromosome length. No other chromosome was morphologically similar to the ASGR-carrier chromosome. One other heteromorphic chromosome also was observed and it may be the homo(eo)log of chromosome 4 since only three structurally similar chromosomes labeled chromosome 4 were found.

The 5S rDNA locus was located adjacent to the centromere in the long arm of four large chromosomes designated chromosome 3 in this tetraploid. After classification of chromosomes based on probe hybridization, five DAPI-stained spreads were analyzed (Table 1) and an ideogram was constructed from the data (Fig. 1c). The length of chromosomes in the genome ranged from 3.4 ± 0.42 μm to 6.1 ± 1.45 μm. DNA contents in each chromosome were estimated based on relative chromosome lengths and the genome size of 1500 Mbp/C (Roche et al. 2002) and ranged from 60 to 108 Mbp. A statistical comparison (*t*-test) of the ASGR-carrier chromosome arms with the other 18S rDNA chromosome type (chromosome 8) showed a significant difference for short arm ($t = 7.36$, $P = 2.62\times10^{-9}$) and total chromosome ($t = 5.42$, $P = 2.08\times10^{-6}$) length but no significant difference for long-arm length ($t = 1.33$, $P = 0.19$). These significant differences support that the ASGR-carrier chromosome is heteromorphic when compared with its presumed homo(eo)logs, all of which have an 18S rDNA locus in common.

Characteristics of the ASGR-carrier chromosome in *C. ciliaris*

Twenty-seven additional mitotic chromosome spreads that showed clear hybridization with BAC clones C001 (containing SCAR Q8) and C101 (containing SCAR ugt197) were analyzed to characterize the ASGR-carrier chromosome in B-12-9. The chromosome characteristics were 2.3 ± 0.36 μm for short-arm length, 3.0 ± 0.92 μm for long-arm length, 5.3 ± 1.17 μm for chromosome length and 1.3 ± 0.32 for arm ratio. There was little difference between these results and the results in Table 1 that were used to construct the ideogram. Distribution of the DAPI fluorescence intensity throughout the ASGR-carrier chromosome is shown in Fig. 1d. DAPI intensity on the short arm was higher than the long arm, which indicates that the short arm is more highly condensed.

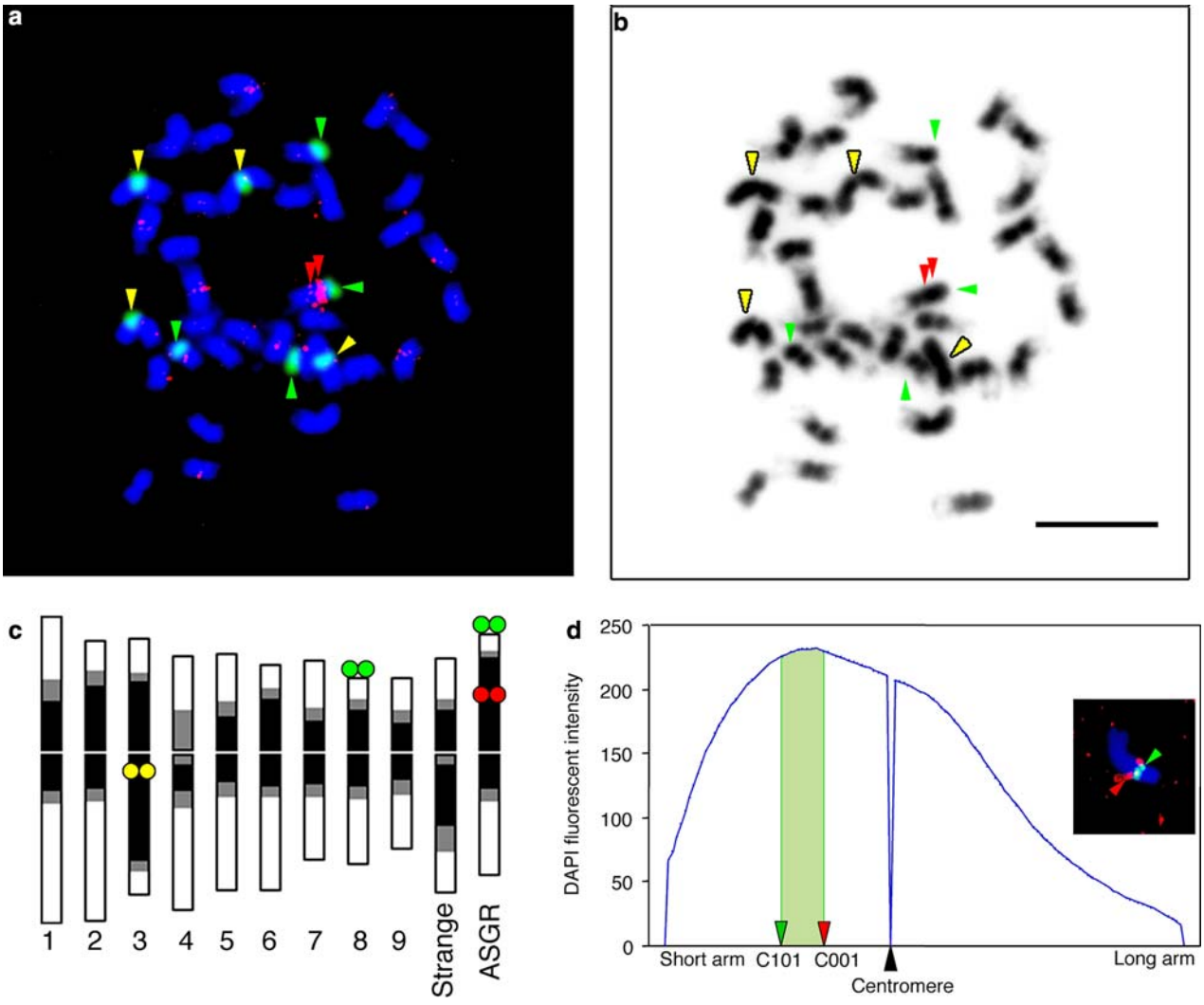

Fig. 1 Physical mapping on mitotic chromosomes in buffelgrass (B-12-9). **a** Mitotic chromosome spread. *Red*, *green*, and *yellow arrowheads* indicate C101, 18S rDNA and 5S rDNA, respectively. **b** Inverted DAPI image of (**a**). **c** Quantitative ideogram. *Red*, *green*, and *yellow circles* indicate C101, 18S rDNA, and 5S rDNA, respectively. **d** DNA distribution pattern in the ASGR-carrier chromosome based on 27 spreads. Image in inset shows an example of the ASGR-carrier chromosome used for analysis. *Red* and *green arrowheads* indicate C101 (ugt197) and C001 (Q8), respectively. *Bars* correspond to 10 μm

C001 and C101 were mapped in B-12-9 at $71 \pm 9.3\%$ and $53 \pm 8.9\%$ from the distal end of the short arm, respectively. This order, the Q8-containing BAC more proximal in position and ugt197 more distal, was inverted in orientation compared with the ASGR in *P. squamulatum* (Akiyama et al. 2004). The distance between the two BAC clones was measured as 0.42 ± 0.20 μm which was estimated to span 11 ± 5.3 Mbp based on the DAPI intensity and the estimation of DNA content in the chromosome.

BAC clones C001, C101, and the 18S rDNA probe were applied to another aposporous accession, Higgins (nine spreads). The ASGR-carrier chromosome in Higgins was slightly shorter than B-12-9 (Table 1), which was more likely due to differences in the mitotic stages sampled than in actual structural differences, thus physical distance between the BACs was not estimated. Nevertheless, the ASGR-carrier chromosome in both genotypes showed similar morphologies, i.e., submetacentric, highly condensed chromatin on the short arm, the same position of the ASGR on the short arm, and the same orientation of BAC-FISH signals.

Comparison of 18S rDNA-bearing chromosomes in B-2S and Higgins

When BAC clones C001, C101, and the 18S rDNA probe were applied to the sexual accession B-2S (six spreads), only the 18S rDNA probe clearly hybridized. Four 18S rDNA-bearing chromosomes were detected in both Higgins and B2-S which was consistent with the observations of B-12-9. Only a single ASGR-carrier chromosome in Higgins displayed signals from BAC clones C001 and C101, while no distinct C001 and C101 signals were detected in B-2S except for very small signals of C101 around most centromeres. No morphological difference was observed among the 18S rDNA-bearing chromo-

Table 1 Characteristics of chromosomes in *C. ciliaris*. Five metaphase spreads were analyzed, thus the number (N) of structurally similar chromosomes analyzed ranged from 5 for the single, heteromorphic ASGR-carrier chromosome to 20 for most other chromosomes in this tetraploid genotype

Accession	Chromosome	N	Length (μm)	SD	Arm ratio (L/S)	SD	DNA content (Mbp)	Note
Apomictic B-12-9	Mitotic							
	1	20	6.1	1.45	1.3	0.30	108	
	2	20	5.6	0.94	1.6	0.35	99	
	3	20	5.1	0.71	1.3	0.18	90	5S rDNA
	4	15	5.1	0.91	1.7	0.50	90	
	5	20	4.7	0.64	1.4	0.29	83	
	6	20	4.5	0.77	1.6	0.45	80	
	7	20	4.0	0.57	1.2	0.25	70	
	8	15	3.7	0.53	1.5	0.33	66	18S rDNA
	9	20	3.4	0.42	1.4	0.44	60	
	Unique	5	4.6	0.46	1.5	0.36	83	Putative chromosome 4 homo(eo)log
	ASGR	5	4.8	0.60	1.1	0.43	85	18S rDNA
	Meiotic							
	ASGR Short arm	13	4.0	0.78	–	–	–	
Apomictic Higgins	Mitotic							
	ASGR	9	4.0	0.96	1.3	0.29	–	18S rDNA
	18S middle	9	3.4	0.51	1.4	0.52	–	18S rDNA
	18S short	18	3.1	0.67	1.6	0.32	–	18S rDNA
Sexual B-2S	18S	24	3.7	0.73	1.5	0.42	–	18S rDNA

somes of B-2S. On the other hand, three types of chromosomes were observed in Higgins, the largest was the ASGR-carrier chromosome, one was mid-sized, and two were relatively short (Table 1). The length and arm ratio of the chromosomes in Higgins were compared statistically by a t-test (Table 2). A significant difference was observed for both parameters between the ASGR-carrier chromosome and the other 18S rDNA-bearing chromosomes of Higgins, although, no significant difference was found between the mid-size and short chromosomes. These results indicate that the ASGR-carrier chromosome in Higgins is also different from the other 18S rDNA-bearing chromosomes, as in B-12-9.

Distribution of BAC clone P602 that contains a partially characterized repetitive sequence

The BAC clone P602 contains an Opie-2-like retroelement that has been shown to hybridize to two chromosome blocks in *P. squamulatum* that flank the hybridization signals from *P. squamulatum* orthologs of C001 and C101 (Akiyama et al. 2004). When P602 was hybridized to mitotic chromosomes of B-12-9, Higgins, and B-2S along with C101, P602 was detected as a very strong signal in the centromeric and pericentromeric region of all chromosomes (Fig. 2a–c) in all accessions. In addition, the ASGR-carrier chromosome showed signal not only near and at the centromere but also along the telomeric region (Fig. 2c). The signal intensity of P602 near the C101 site of hybridization was not strong and C101 appeared to localize to a gap between repetitive blocks.

Physical mapping of the apospory-linked BACs on pachytene chromosomes of B-12-9

The BAC clones containing SCARs Q8 (C001, C013, and P109) and ugt197 (C101 and P208) were mapped on pachytene chromosomes of B-12-9. Even at the pachytene stage, the ASGR-carrier chromosome showed a highly condensed structure and the two signals were no better resolved at pachytene than in mitotic chromosomes (Fig. 2d). Signals from the three Q8-containing BACs overlapped at the same position. The two ugt197-containing BACs hybridized to the same chromosomal location except that duplicate sites in close proximity to one another sometimes could be observed as with mitotic chromosomes.

Since chromosome extension for the ASGR did not appear to be much greater in pachytene of meiosis than in mitosis, the total short-arm length was measured in 13

Table 2 Comparison among 18S rDNA bearing mitotic metaphase chromosomes in apomictic Higgins

Chromosome type	ASGR-carrier	Mid-size 18S	Short 18S
AGSR-carrier		$t = 1.72$, $P = 0.09^{a,*}$	$t = 2.15$, $P = 0.035^{a,**}$
Mid-size 18S	$t = 2.49$, $P = 0.018^{b,**}$		$t = 0.80$, $P = 0.42^{a}$
Short 18S	$t = 3.96$, $P = 0.0002^{b,***}$	$t = 1.39$, $P = 0.17^{b}$	

[a]Arm ratio comparison
[b]Length comparison
$^{*}P < 0.1$, $^{**}P < 0.05\%$, $^{***}P < 0.01\%$

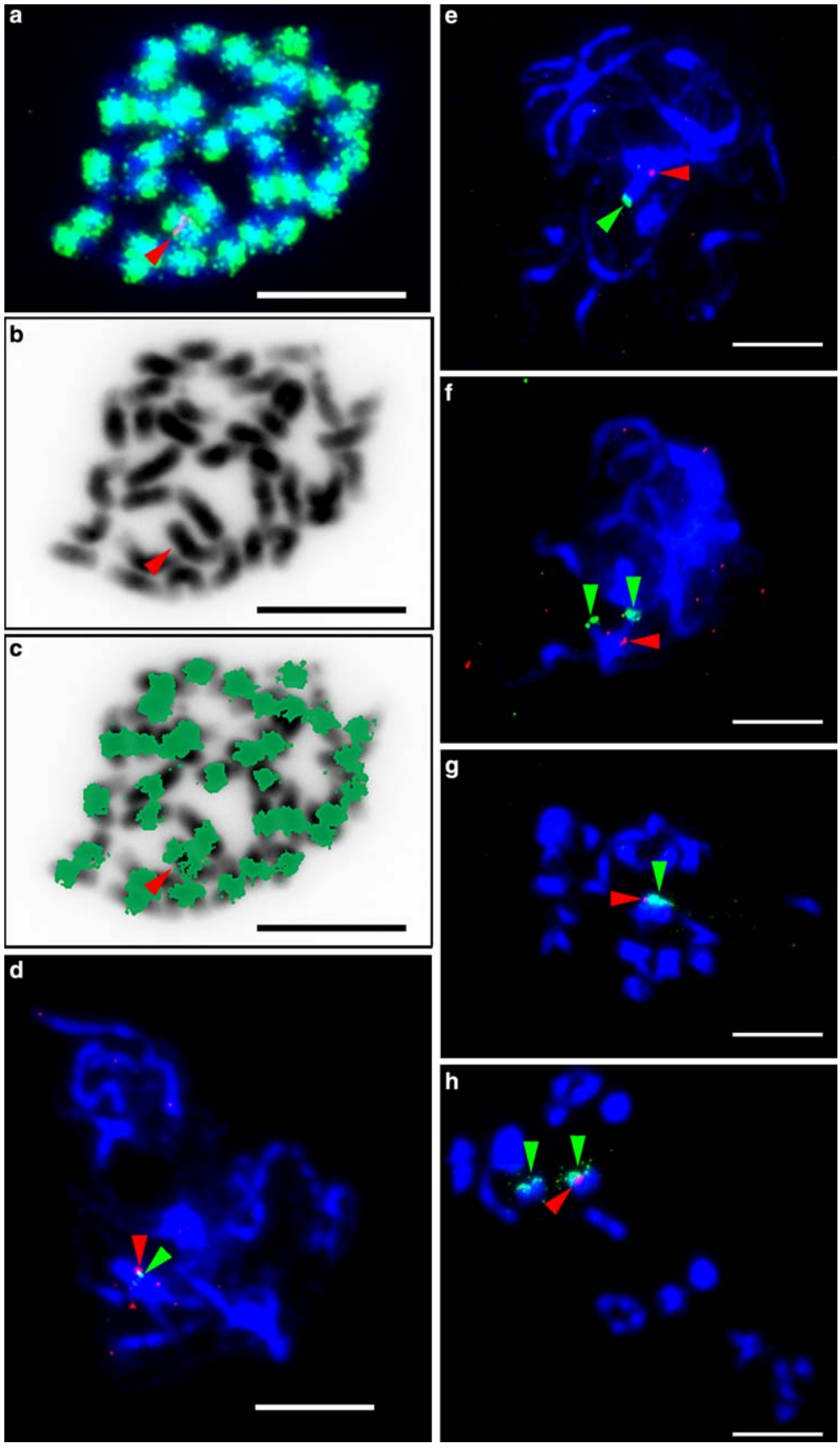

Fig. 2 Fluorescence in situ hybridization analysis of buffelgrass (B-12-9) chromosomes. **a** Mitotic chromosome spread painted by P602. **b** Inverted DAPI image of (**a**). **c** Highlighted P602 region on image of (b). **d** Pachytene chromosome spread mapped by low-copy, ASGR-linked BACs. *Red* and *green arrowheads* indicate C101 and C001, respectively. (e)-(h) Meiotic chromosome spreads. Red and green arrowheads indicate C101 and 18S rDNA, respectively; (e)(f) at pachytene stage; (g)(h) at diakinesis stage. *Bars* correspond to 10 μm

spreads after dual hybridization of the 18S rDNA and a centromeric probe (Goel et al. 2003) (Table 1). The length at pachytene was 3.98 ± 0.78 μm which was only 1.7X that of the short arm of the ASGR-carrier chromosome at mitosis. For the other 18S-bearing chromosomes, the extension was 2.7X that of mitotic

chromosomes. This result indicates that the short arm of the ASGR-carrier chromosome is highly condensed even at the pachytene stage. This difference in degree of extension between mitotic and meiotic chromosomes is the smallest previously reported in plants (5-fold in *P. squamulatum*; Akiyama et al. 2004; 7-fold in rye, and 40-fold in rice, de Jong et al. 1999).

Association of the ASGR-carrier chromosome with other 18S rDNA-bearing chromosomes at meiosis

We investigated pairing of the 18S rDNA-bearing chromosomes at meiosis for B-12-9, Higgins and B-2S (Fig. 2e–h; Table 3). In all genotypes, a single integrated signal on a thick chromatin region was most frequently observed at the pachytene to diplotene stages (Fig. 2e) and persisted in 30–60% of the spreads at diakinesis (Table 3, Fig. 2g). When two signals were occasionally observed at the pachytene to diplotene stages, they were positioned adjacent to one another and all four chromosomes appeared to be joined together proximal to the rDNA locus (Fig. 2f). The number of spreads with two separate signals became more frequent (40–70%) at diakinesis (Table 3, Fig. 2h). Signal from C101 was clearly observed on only one of the 18S rDNA-bearing chromosomes in the apomictic genotypes, B-12–9, and Higgins. These results suggest that the ASGR-carrier chromosome and at least one other 18S rDNA-bearing chromosome are homologous since they can pair and likely undergo synapsis and recombination, at least outside of the ASGR itself. The association of all four 18S rDNA-bearing chromosomes is clearly predominant in early meiosis I and persists in some cases through diakinesis thus raising the question of whether pairing is always random or tends to be preferential.

Shotgun FISH for finding the centromere-specific sequence in P208

BAC clone P208 and an orthologous BAC C101, had been observed to hybridize to centromeric regions of both species. We constructed a shotgun library (~2 kb insert size) from P208 and carried out FISH with six pools of 10 randomly selected subclones each. One pool produced signal on the centromeres; therefore, all 10 subclones from the pool were hybridized individually. Only one subclone (P208D1) showed signal on the centromeres. Moreover, the probe showed a small signal on the ASGR in the same position as P208 (Fig. 3). The clone was completely sequenced and used to query Genbank (nr) by BLASTX. The subclone was 2060 bp in length and one end (637 bp) showed similarity to three high-scoring segment pairs from the gag-pol region of the centromeric retrotransposon of maize, CRM (AAM94350.1; E value = 1e-32) and rice (AAQ56339; E-value = 7e-35) across 233–236 amino acids (30–59% identity).

Discussion

The ASGR-carrier chromosome in *C. ciliaris*

Based on chi-square analysis of segregating populations, Sherwood et al. (1994) proposed that B-12-9 had one allele for apospory and that Higgins had two. Our FISH results support the presence of a single dose of the ASGR in B-12-9 and Higgins but no ASGR in B-2S. Three possible explanations for this discrepancy could be that (1) the second dose of the apospory allele could be located within an ASGR of minimal size that does not contain the BACs used for FISH; (2) the models for inheritance proposed cannot account for the variation in lethality that may result from recombination on the ASGR-carrier chromosome arm followed by distorted segregation; and (3) environmental factors affected crossing efficiency. The first explanation seems unlikely since all apomictic progeny from a cross with Higgins as male parent also carried the apomixis-linked markers used to isolate the BACs. If an additional but smaller ASGR were present on one homolog that carried an allele for apospory, some of the apomictic progeny should have been lacking some of the apomixis-linked

Table 3 Chromosome pairing at meiosis in *C. ciliaris*

Accession	Stage		The number of 18S rDNA signals		Total
			1	2	
ApomicticB-12-9	Pachytene to diplotene	N	58	1	59
		%	98	2	
	Diakinesis	N	6	14	20
		%	30	70	
ApomicticHiggins	Pachytene to diplotene	N	52	2	54
		%	96	4	
	Diakinesis	N	20	13	33
		%	61	39	
Sexual B-2S	Pachytene to diplotene	N	49	7	56
		%	88	12	
	Diakinesis	N	16	12	28
		%	57	43	

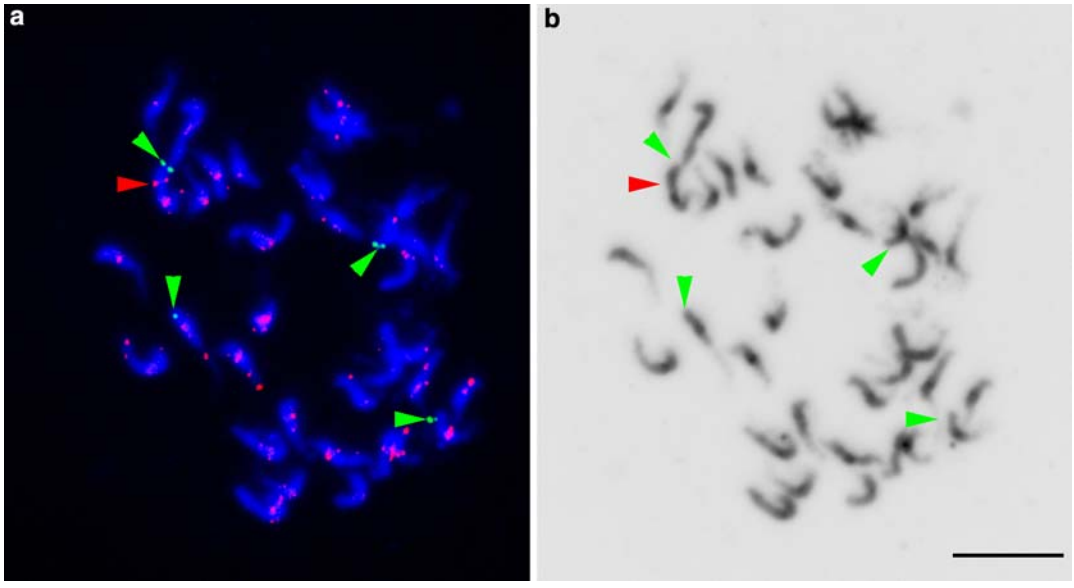

Fig. 3 Physical mapping of the CRM-related P208 subclone in buffelgrass B-12-9. **a** Mitotic chromosome spread at prometaphase. *Red* and *green arrows* indicate the ASGR and 18S rDNA, respectively. **b** Inverted DAPI image. *Bar* corresponds to 10 μm

markers. No homozygous aposporous apomicts have been found in nature (Nogler 1984). In addition, our survey of apomictic genotypes from 10 *Pennisetum* species and 2 *Cenchrus* species has identified only one that has two ASGR-carrier chromosomes in its genome (Akiyama et al. unpublished).

C101 and P208, the ugt197-containing BACs that clearly hybridize to the ASGR, also gave very small signals at the centromeric region of all chromosomes in both apomictic and sexual genotypes of *C. ciliaris*. Shotgun-FISH revealed that P208 apparently contains sequence with similarity to a centromeric retrotransposon from maize (CRM; Zhong et al. 2002). The CRM elements are strictly localized to the centromere in maize and are conserved among cereals (Mroczek and Dawe 2003). Since we have not sequenced a full-length, CRM-like element from the ASGR of buffelgrass, it is possible that it could be a related, non-centromere-specific retroelement similar to the *Tekay/Prem-1* family in maize, which is related to CR elements (Mroczek and Dawe 2003). However, *Tekay/Prem-1* is dispersed throughout the genome of maize contrary to our observation that the CRM-like element from *P. squamulatum* showed localized FISH signals in buffelgrass. If this CRM-like element is centromeric in origin, the localized FISH signal in the ASGR could be due to the translocation of an ancestral ASGR, along with centromere-related sequences, from a more centromere-proximal position to its current position in buffelgrass. Alternatively, the CRM-like element could have arisen due to localized transposition. The readily detectable CRM-like signal at the ASGR indicates that there are multiple elements in this region since it is unlikely that strong signal would have been observed with a single-copy 2 kb probe. Goel et al. (2003) showed that a BAC clone containing a 160 bp *KpnI*, centromere-specific repeat from *P. squamulatum* (Ingham et al. 1993; Kamm et al. 1994), hybridized only to centromeres of all chromosomes including the ASGR-carrier chromosome in buffelgrass. If this satellite repeat is required for functionality, this result suggests that the ASGR in buffelgrass does not contain a functional centromere.

Comparison of the ASGR between *C. ciliaris* and *P. squamulatum*

We recently showed that BACs containing SCARs ugt197 and Q8 were located on the same chromosome in *C. ciliaris*, but they could not be ordered because of overlapping of signals (Goel et al. 2003). We now show that these signals can be separated on mitotic chromosomes at prometaphase stage and on pachytene chromosomes. The order of the BACs in *C. ciliaris* was inverted compared with *P. squamulatum* (Akiyama et al. 2004); however, the distance between the BACs remained approximately the same at 11–13 Mbp in both species. Our results indicate that the loci surrounding ugt197 and Q8 are similar in both species because clear signals in the same positions could be detected by heterologous BAC hybridization.

A distinct difference between the two species was observed in the distribution of sequence hybridizing to the BAC P602. Signal from this BAC was particularly abundant on the distal half of the short arm of the ASGR-carrier chromosome in *P. squamulatum*, but little signal was observed over the remainder of the genome (Ozias-Akins et al. 2003; Akiyama et al. 2004). In *C. ciliaris*, however, P602 hybridized across the genome especially across centromeric regions, which are highly condensed. Only the ASGR-carrier chromosome also showed P602 signal at the telomeric region, which also was highly condensed like the centromeric region. The

short arm of the 18S rDNA- and ASGR-bearing chromosome was not elongated even at pachytene, which may reflect a heterochromatin composition. The hybridization patterns of P602, C101, and C001 indicate that a structural change (inversion) in the ASGR-carrier chromosome occurred during the divergence of *C. ciliaris* and *P. squamulatum* and that the abundance of the repetitive sequences at the ASGR in *P. squamulatum* may be a reflection of the ancestral origin of the ASGR rather than recent accumulation of retroelements.

Pairing between the ASGR-carrier chromosome and homo(eo)logous chromosomes

The ASGR-carrier chromosome is heteromorphic, approximately 20 Mbp larger, when compared with its presumed homo(eo)logs that also have rDNA loci. The difference in size is mainly due to the short arm where the ASGR is located. Jessup et al. (2002) reported that the ASGR-carrier chromosome showed preferential pairing with a homologous chromosome based on molecular map construction. However, we observed that the 18S rDNA loci on each of the short arms aggregated at early meiosis and this aggregation persisted in many cases into diakinesis. It is possible that these chromosomes are pairing randomly and in some cases form quadrivalents. Other cytological investigations have reported the formation of 1–3 quadrivalents in some buffelgrass accessions (Visser et al. 1998b). The strong association among all four 18S rDNA chromosomes that we observed during early meiosis could be due in part to the activity of the 18S rDNA loci, but more mapping data would be necessary to unequivocally argue for preferential pairing. Even though the ASGR-carrier chromosome is heteromorphic, its recognition of homologs may not be impaired. Further investigation of synapsis would have to be carried out with higher resolution techniques.

Inert recombination in the ASGR

Lack of recombination in the ASGR of *P. squamulatum* has been shown and is a serious obstacle for the construction of a genetic map (Ozias-Akins et al. 1998). *C. ciliaris* has been used for linkage map construction in order to position the gene(s) for apospory (Gustine et al. 1997; Roche et al. 1999; Jessup et al. 2002, 2003). Jessup et al. (2002) reported that the distance between markers that most closely flanked the apospory gene was 10 cM. The resolution of a linkage map is subject to variation in the level of recombination and genetic distance is not closely correlated with physical distance. It has been shown that recombination is reduced in retrotransposon-rich regions (Cold Spring Harbor Laboratory 2000; Fu et al. 2002; Yao et al. 2002) and in pericentromeric regions (Islam-Faridi et al. 2002; Künzel et al. 2000; Kurata et al. 2002; Tanksley et al. 1992) both of which are frequently heterochromatic. We showed in this study for *C. ciliaris* that (1) the ASGR was located on a highly condensed chromatin region at mitosis, (2) decondensation of the short arm of the ASGR-carrier chromosome at pachytene was extremely small, and (3) the ASGR-carrier chromosome includes an abundant repetitive DNA family. These results are in accordance with our previous report for *P. squamulatum* (Akiyama et al. 2004). There is at least an 11 Mbp region (between BACs C001 and C101) where recombination has not been observed in either our study (Roche et al. 1999) or Jessup et al. (2002). These results imply that there is a serious limitation in *C. ciliaris* as well as in *P. squamulatum*, for the construction of a detailed genetic map for the ASGR.

Acknowledgements We are grateful for the technical assistance of Anne Bell, Evelyn Perry, Gunawati Gunawan, and Jacolyn Merriman. We appreciate Drs. Joann A. Conner and Shailendra Goel for helpful discussion and comments. We thank Dr. Nobuko Ohmido for providing 5S rDNA plasmid and Prof. Kiichi Fukui and Mr. Seiji Kato for technical advice to make the quantitative ideogram. This work was supported by the USDA National Research Initiative (award no. 02-35301-12283) and the National Science Foundation (award no. 0115911).

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