Theor Appl Genet (2002) 104:804–812
DOI 10.1007/s00122-001-0795-y

D. Roche · J. A. Conner · M. A. Budiman · D. Frisch
R. Wing · W. W. Hanna · P. Ozias-Akins

Construction of BAC libraries from two apomictic grasses to study the microcolinearity of their apospory-specific genomic regions

Received: 25 March 2001 / Accepted: 20 June 2001 / Published online: 6 February 2002

Abstract We have constructed bacterial artificial chromosome (BAC) libraries from two grass species that reproduce by apospory, a form of gametophytic apomixis. The library of an apomictic polyhaploid genotype (line MS228-20, with a 2C genome size of approximately 4,500 Mbp) derived from a cross between the obligate apomict, *Pennisetum squamulatum*, and pearl millet (*P. glaucum*) comprises 118,272 clones with an average insert size of 82 kb. The library of buffelgrass (*Cenchrus ciliaris*, apomictic line B-12-9, with a 2C genome size of approximately 3,000 Mbp) contains 68,736 clones with an average insert size of 109 kb. Based on the genome sizes of these two lines and correcting for the number for false-positive and organellar clones, library coverages were found to be 3.7 and 4.8 haploid genome equivalents for MS 228-20 and B12-9, respectively. Both libraries were screened by hybridization with six SCARs (sequence-characterized amplified regions), whose tight linkage in a single apospory-specific genomic region had been previously demonstrated in both species. Analysis of these BAC clones indicated that some of the SCAR markers are actually amplifying duplicated regions linked in coupling in both genomes and that restriction enzyme mapping will be necessary to sort out the duplications.

Communicated by D. Hoisington

D. Roche · W.W. Hanna
Crop Genetics and Breeding, USDA-ARS,
Coastal Plain Experiment Station, Tifton, GA 31793, USA

J.A. Conner · P. Ozias-Akins (✉)
Department of Horticulture, University of Georgia –
Tifton Campus, Tifton GA 31793-0748, USA
e-mail: Ozias@tifton.cpes.peachnet.edu
Fax: +1-229-3863356

M.A. Budiman · D. Frisch · R. Wing
Clemson University Genomics Institute, Jordan Hall, Clemson,
SC 29634, USA

Present address:
D. Roche, Plants, Soils and Biometerology Department,
UMC 4820, Utah State University, Logan, UT 84322, USA

Keywords Apomixis · Apospory · BAC library · Colinearity · *Pennisetum*

Introduction

Apomixis is a naturally occurring mode of reproduction in which plants, regardless of their level of heterozygosity, clonally propagate themselves through seeds (Asker and Jerling 1992). The understanding as well as the control of the mechanisms underlying apomixis could have a remarkable impact on crop breeding and seed production (Hanna 1995). We have concentrated our research efforts on one form of gametophytic apomixis, pseudogamous apospory, in which unreduced embryo sacs originate from somatic nucellar cells (Nogler 1984). The 2*n* egg cell in each embryo sac is not fertilized by a sperm cell but nevertheless develops parthenogenetically to form an embryo whose genotype is identical to that of the seed-bearing plant. We have recently shown that a single apospory-specific genomic region (ASGR) is sufficient for the expression of apospory in two grasses, *Pennisetum squamulatum* Fresen and *Cenchrus ciliaris* L. [syn. *P. ciliare* (L.) Link; buffelgrass], both of African origin (Ozias-Akins et al. 1998; Roche et al. 1999). ASGR-linked molecular markers are conserved between both species (Lubbers et al. 1994; Roche et al. 1999). Unfortunately, recombination between these markers has not been detected, thereby hampering the prospect of finer genetic mapping in order to clone the gene(s) for apospory using positional information. Given the conservation of markers within the ASGR of both species, we decided to analyze the microcolinearity of this region in order to identify DNA regions highly conserved between the two species. Analysis of gene content within the conserved regions of the ASGR from both species would provide candidate gene targets. A necessary prerequisite for high-resolution comparative analysis at the structural level is the construction of large-insert genomic libraries.

For all organisms, bacterial artificial chromosome (BAC) libraries have become the most efficient tool by

which to clone whole genomes since the demonstration by Shizuya et al. (1992) that a bacterial plasmid could be used to clone large DNA inserts. Insert sizes often are in the average range of 80–150 kb, thus three to four times larger than those of cosmid libraries (25–45 kb) (Sambrook et al. 1989). Although BAC inserts are significantly smaller than those in yeast artificial chromosomes (YAC libraries; average of 500 kb) (Burke et al. 1987), the production of BAC libraries, as well as their utilization, present many advantages over YAC libraries, including the ease of DNA isolation, the low frequency of chimeras, and insert stability (Cai et al. 1995). We document here the construction of BAC libraries from an apomictic polyhaploid line derived from an F_1 hybrid between pearl millet [*Pennisetum glaucum* (L.) R. Br.] and *P. squamulatum* (Dujardin and Hanna 1986) and an apomictic buffelgrass (*Cenchrus ciliaris*).

Materials and methods

Plant material

Apomictic line MS228-20 was germinated from seeds of an open-pollinated apomictic polyhaploid F_1 line derived from a cross between *Pennisetum glaucum* (pearl millet) and *P. squamulatum* (Dujardin and Hanna 1986), hereafter referred to as simply "polyhaploid". The presence of the ASGR in the polyhaploid was confirmed by the polymerase chain reaction (PCR) using sequence-characterized amplified regions (SCARs) as outlined in Ozias-Akins et al. (1998). Other sexual and apomictic F_1 individuals (290-105, 290-124) from the mapping population (Ozias-Akins et al. 1998) were used during restriction fragment length polymorphism (RFLP) analysis. Apomictic line B-12-9 of bufflegrass (*Cenchrus ciliaris*) was provided by R.T. Sherwood and D.L. Gustine, USDA-ARS, University Park, Penn. The pedigree of this line can be found in Sherwood et al. (1994). A sexual buffelgrass genotype, B-2s, received from the same source, had been previously used as the sexual parent in segregating crosses to map the apomixis locus (Gustine et al. 1997; Roche et al. 1999). Plants from species and hybrids were propagated vegetatively for several years. DNA isolations were conducted as previously described (Ozias-Akins et al. 1998).

Nuclear DNA content analysis of different germplasm lines

A piece of young leaf was chopped with a razor blade in buffer according to Otto (1994). The chopped tissue was diluted with 3 ml of 0.4 *M* Na_2 HPO_4 containing the DNA-specific fluorochrome DAPI (0.2 mg/100 ml) and filtered through a 40-µm sieve. Suspended nuclei (10,000 per sample) were analyzed on a PAS-III flow cytometer (Partec, Munster, Germany) equipped with a 100-W high-pressure mercury lamp.

BAC library construction

Isolation of nuclei for preparation of megabase DNA

Plants were etiolated for 72 h, after which fresh leaves and stems were harvested to isolate nuclei for megabase DNA preparation. Ten to twenty grams of tissue was ground in liquid nitrogen in 2- to 3-g aliquots. Liquid nitrogen was never added to partially ground tissue to prevent nuclei damage. A single homogenization buffer (Zhang et al. 1995) containing 0.5% triton X-100 was used throughout the nuclei preparation. The powdered tissue was mixed with homogenization buffer (10 ml per gram of tissue) and slowly stirred on ice for 15–20 min. The homogenate was successively filtered through three nylon sieves of 295, 105 and 60 µm into 250-ml centrifuge bottles and spun at 1,800 *g* for 10 min. The supernatant was discarded, and the pellet was gently resuspended with a small paintbrush in 30 ml of homogenization buffer, then filtered through Miracloth (CN Biosciences, Darmstadt, Germany) when necessary to remove clumps of nuclei. A light spin at 60 *g* for 2 min was used to further remove nuclei clumps. The supernatant was spun at 2,000 rpm for 5 min to collect the nuclei, and the pellet was gently resuspended in 15 ml of cold homogenization buffer. Three further nuclei washes were conducted with the same conditions for centrifugation and resuspension. All filtrations and centrifugations described above were carried out at 0 °–4 °C. The final nuclei pellet was warmed at 45 °C and resuspended at a final concentration of 10^7 nuclei per milliliter with an equal volume of 1.6% Incert Agarose (FMC, Rockland, Md.) in homogenization buffer prewarmed at 45 °C. Nuclei-agarose plugs of 100 µl each were made on ice in a plastic mold.

Preparation of cloning vector

The single-copy BAC vector, pBeloBAC11, was prepared as described by Woo et al. (1994) omitting purification on a cesium chloride density gradient.

Size-selection and cloning of megabase DNA

Nuclei in agarose plugs were lysed for 48 h, and then plugs were washed and equilibrated in buffer as described by Zhang et al. (1995). Agarose plugs were divided into thirds, and then each of the approximately 30-µl segments was finely chopped and equilibrated on ice for 45 min in *Hin*dIII reaction buffer. Megabase DNA was digested by adding 2.5–5 U *Hin*dIII restriction enzyme (NEB, Beverly, Mass.) in a total volume of 50 µl, incubating the complete reaction mix on ice for 45 min, followed by incubation for 20 min at 37 °C. Partially digested DNA was electrophoretically separated on 0.9% agarose CHEF gels in 0.5×TBE at 12 °–15 °C (first size selection: 17 h, 5.0 V/cm, constant switch at 75 s; second size selection: 15 h, 5.0 V/cm, constant switch at 5 s). Gel slices containing DNA of different molecular weights were electroeluted in 1×TAE at 4 °C for 2 h in dialysis membranes (12,000–14,000 mwco). Following gel quantification of an ethidium bromide-stained aliquot of the electroeluted DNA, 80–150 ng of plant DNA was mixed with 20 ng of *Hin*dIII-cut and dephosphorylated BAC vector and ligated at 16 °C overnight in a total volume of 100 µl in the presence of 10 U T4 DNA ligase and under the manufacturer's reaction buffer conditions (Promega, Madison, Wis.). Following a desalting incubation of 2 h on ice in a 1.5-ml microcentrifuge tube containing 1.0 ml 100 m*M* glucose and 1% LE agarose (FMC), 1.0 µl from each ligation was used to transform 20 µl of competent cells (electrocompetent DH10B, Life Technologies, Rockville, Md.). Electroporation was carried out using an EC600 apparatus (BTX Corp, San Diego, Calif.) at 1.5 kV, 129 ohms, a pulse of 4.85–4.95 ms, and 1-mm gap cuvettes. Electroporated cells were immediately diluted in 1 ml of SOC medium (Sambrook et al. 1989) and incubated at 200 rpm at 37 °C for 1 h. Dimethylsulfoxide (DMSO) was added to the cells (7% v/v final concentration) before freezing at –80 °C. The addition of DMSO maintained the viability of the transformed cells for several months (data not shown). Hence, loss of titer was conveniently prevented during clone sizing and characterization of each ligation. Transformed cells were plated on 200 ml of selective medium (LB; Luria-Bertani medium with 15 µg/ml chloramphenicol, 0.55 m*M* IPTG and 80 µg/l of X-gal) poured into Q-trays (Genetix, Queensway, UK). Following two 24-h incubations in the dark (first 24-h period at 37 °C, second at room temperature), white recombinant colonies were picked robotically (Q-Bot, Genetix) and stored in 384-well microtiter plates (Genetix) filled with 65 µl of freeze broth (Woo et al. 1994) per well. Recombinant colonies which were avoided by the robot because they were either too close to other colonies or to the tray edge were picked manually

Table 1 Nuclear DNA contents estimated from DAPI-stained nuclei

	2C DNA content (Mbp)		Number of haploid genome equivalents in BAC libraries
	Present estimate	Previous estimates	
Oryza sativa		980[c]	
Pennisetum glaucum (2×)	3,900	2,150[a]; 4,600[b]; 4,700–5,200[c]	
Pennisetum glaucum (4×)	7,800		
Pennisetum squamulatum	10,300	9,400[c]	
F_1 290-124	9,300		
Polyhaploid	4,500	4,700[d]	3.6–3.8
Cenchrus ciliaris	3,000	2,600[c]	4.8–5.5

[a] Marie and Brown (1993)
[b] Martel et al. (1997)
[c] C-value database at www.rbgkew.org.uk
[d] Extrapolated from the literature values for *P. glaucum*[b] and *P. squamulatum*[c]

with sterile disposable pipette tips. Microtiter plates were incubated overnight at 37 °C and robotically (Q-Bot) duplicated for storage in different freezers.

Characterization of BAC clones

Individual BAC clones were grown overnight at 37 °C in a total volume of 3 ml LB medium containing 15 µg/ml chloramphenicol. Isolation of the plasmid was carried out using a standard alkaline-lysis method (Sambrook et al. 1989). The final pellet was dissolved in a total of 15 µl of TE buffer, from which 5 µl was used for each restriction enzyme digestion prior to electrophoretic analysis.

Screening of BAC libraries

High-density colony filters were prepared with the Genetix Q-Bot as described in Tomkins et al. (1999a). Six sets of six and four filters each were made for the polyhaploid and buffelgrass libraries, respectively. Radioactive decay (not stripping) was used between two successive hybridizations of the same filter. Radiolabeling of probes was done by PCR-incorporation of [^{32} P] using SCAR primers and SCAR clones as template DNAs (Ozias-Akins et al. 1998). Hybridization and identification of addresses for positive clones were performed as indicated at www.genome.clemson.edu/groups/bac/protocols.

Chloroplast and mitochondrial DNA probes

A segment of chloroplast (cp) DNA was amplified by PCR from total DNA of *P. squamulatum* and buffelgrass with the primer combination *trn*H and *trn*K (Demesure et al. 1995). Both species yielded similar 1,900-bp products that were radiolabeled. Total mitochondrial (mt) DNA from *P. glaucum* was isolated from a cell culture line (Ozias-Akins et al. 1987) and labeled with [^{32} P] by random priming.

Fingerprinting analysis and contig assembly

A subset of the clones identified in the library screens were fingerprinted at CUGI (Clemson University Genomics Institute). From the polyhaploid library this included: 3-A14M clones (all ASGR-linked), 8-Q8M clones (all ASGR-linked; 5 PCR-positive, 3 PCR-negative), 7-UGT197 clones (7 ASGR-linked), and 9-O7M clones (6 ASGR-linked, 3 not ASGR-linked). From the buffelgrass library this included: 7-Q8M clones (all ASGR-linked; 4 PCR-positive, 3 PCR-negative), 7-UGT197 clones (all ASGR-linked), and 6-O7M clones (none ASGR-linked). BAC clones were digested with *Hin*dIII, run on an agarose gel, and imaged at CUGI. Band calling and contig building were accomplished using the Image (version 3.10, Sanger Centre, UK) and FPC (version 4.6, CUGI) software with Linux. Parameters used in the FPC analysis were a fixed tolerance value of 7 and a cutoff score of 10^{-12}.

Results

Analysis of genome sizes

The genome size of *Pennisetum glaucum* [syn. *P. americanum* (L.) Leeke and *P. typhoides* (Burm.) Stapf et Hubb.] has been estimated previously to range from 2,100 Mbp to 5,200 Mbp (Marie and Brown 1993; Bennett and Leitch 1997; Martel et al. 1997). Because of the variability reported in the literature, we chose to carry out our own estimates of DNA content using flow cytometry of DAPI-stained nuclei with rice *(Oryza sativa* cv. Lemont) as an internal standard for diploid pearl millet. Table 1 shows the DNA contents of all materials estimated in this study based on their 2C peaks of relative fluorescence intensity. DNA contents of both buffelgrass genotypes (B-2s and B-12-9) were the same, as estimated using diploid pearl millet as the standard for comparison. Genome sizes of the polyhaploid and *P. squamulatum* were estimated using tetraploid pearl millet as the standard. Tetraploid pearl millet showed exactly twice the DNA content of diploid pearl millet when both were run together. Since the genome size of *P. squamulatum* is approximately 10 billion bp it would have been a major undertaking to obtain a representative BAC library of this species. Thus, we chose a 21-chromosome polyhaploid line that was spontaneously derived from an apomictic F_1 individual (Dujardin and Hanna 1986) and was shown to contain all markers for the ASGR from *P. squamulatum*. This line contains seven pearl millet (1×) and 14 *P. squamulatum* (1.5×) chromosomes, one of which represents the apospory linkage group. Upon screening of the reproductive phenotype and analysis of ASGR-linked molecular markers (data not shown), the polyhaploid line was found to display similar results to any other apomictic F_1 individual produced by the cross between tetraploid pearl millet and *P. squamulatum* (Ozias-Akins et al. 1998). Thus, in the polyhaploid, one copy of the ASGR is represented in only half the genome size contained in any F_1 individual, and the number of BAC clones that are required for 1×genome coverage is reduced by half. Even though the ASGR is simplex in these plants, other loci in the polyhaploid or buffelgrass libraries are likely to be represented by multiple alleles; thus, for the calculation of genome coverage we followed the standard of 1×coverage being equivalent to a 1C value.

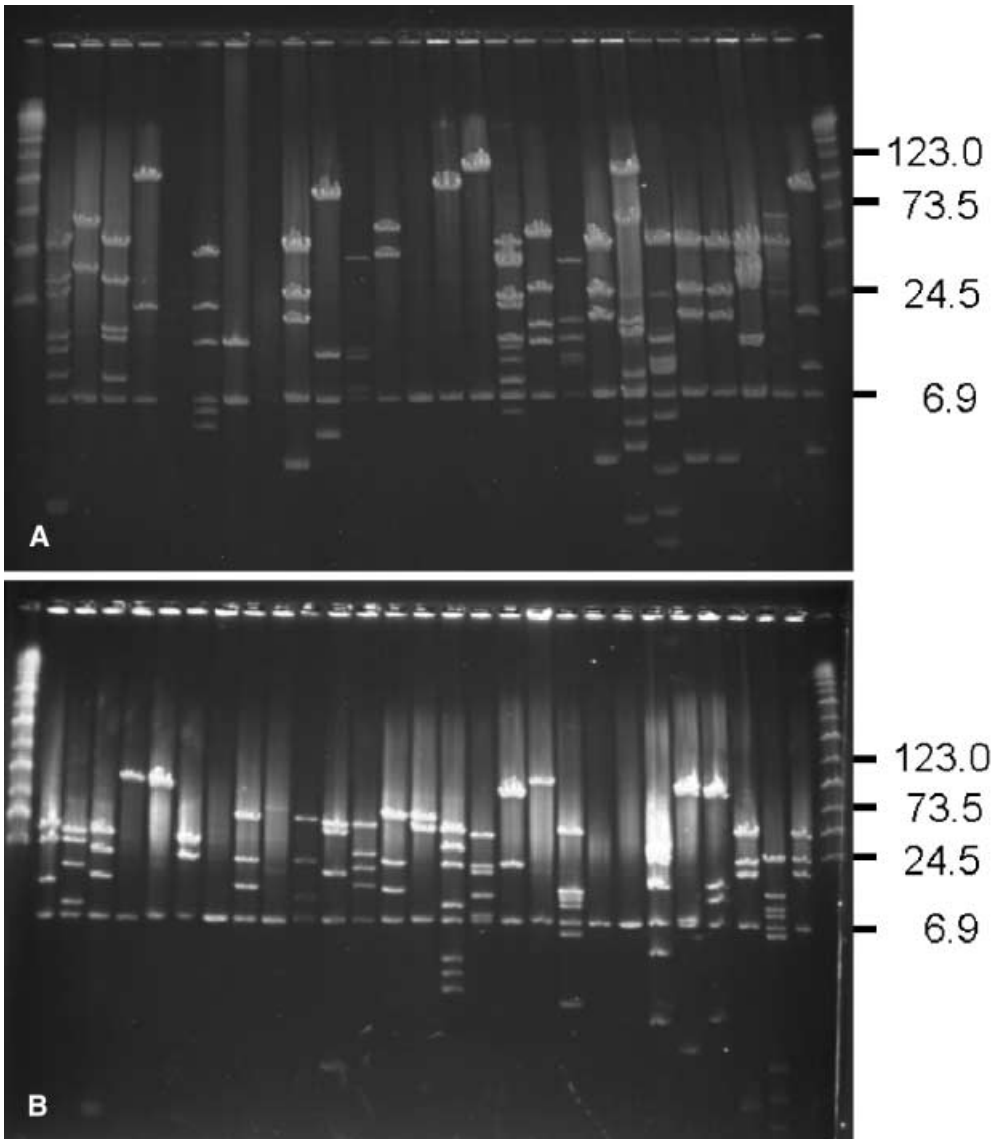

Fig. 1 Analysis of BAC clones by PFG electrophoresis. Ethidium bromide-stained CHEF gels of randomly picked recombinant BAC clones from the buffelgrass (**A**) and polyhaploid (**B**) libraries digested with *Not*I. The *first* and *last lanes* of both gels contain a lambda concatemer marker (NEB). Fragment sizes (in kilobases) are indicated on the *right-hand side* of each panel

Construction and characterization of the libraries

The polyhaploid library contains 118,272 clones with an average insert-size of 82 kb (*n*=l00), and 12% of these clones are false-positives (clones with no insert). The buffelgrass BAC library includes 68,736 clones with an average insert size of 109 kb (*n*=l05) and a small fraction (3%) of false-positives. To estimate the representation of cpDNA in both libraries we hybridized one high-density filter from each library (18,432 clones) with a 1,900-bp fragment amplified between the *trn*H and *trn*K primers (Demesure et al. 1995). Correcting for the insert size of the libraries and for an approximate chloroplast genome size in *P. glaucum* of 120 kb (Smith et al. 1987), an estimated 0.1% and 0.7% of clones are of chloroplast origin in the polyhaploid and buffelgrass libraries, respectively. The proportion of mitochondrial clones were estimated at 0.2% and 0.5% in polyhaploid and buffelgrass libraries, respectively. Taking into consideration false-positives, chloroplast, and mitochondrial clones, as well as present and previous estimates of genome sizes, the coverage of the polyhaploid genome is 3.6–3.8 haploid genome equivalents and the buffelgrass genome is 4.8–5.5 haploid genome equivalents. Random samples of BAC clones from both species are shown in Fig. 1.

The construction of libraries from these two grasses was challenging. For the polyhaploid line, a relatively non-vigorous, vegetatively propagated plant, we performed more than 200 ligations with 20–30 different size selections of megabase DNA and were never successful at exceeding 92 kb as the average insert-size. A majority of the ligations resulted in few total colonies with a high representation of white, false-positive clones containing no inserts (up to l00%, variable with different cloning attempts). Analysis of some false-positive clones with digestion by restriction enzymes and gel electrophoresis revealed partial degradation of the cloning vector (Fig. 2). Deletions of 1–2 kb of the

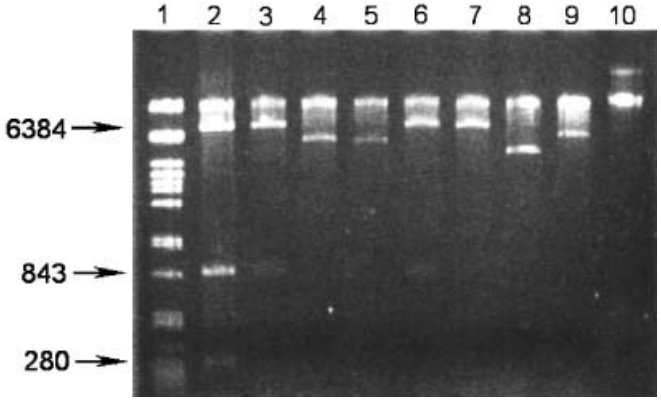

Fig. 2 Degradation of *Hin*dIII-cut and dephosphorylated pBelo-BAC11. *Lanes: 1 Pst*I-digested lambda DNA, *2* intact pBelo-BAC11 vector (isolated from a blue colony) cut with *Sal*I and yielding the predicted size fragments of 6,384 bp, 843 bp, and 280 bp (*arrows*), 3–7 individual false-positive clones digested with *Sal*I; the expected 280-bp fragment that contains the *Hin*dIII cloning site was absent in these clones, while the adjacent 843-bp fragment was present in only *lanes 3* and *6*. Deletions of 1–2 kb of the BAC vector were apparent in *lanes 4, 5* and *7–9*. *Lane 10* undigested pBeloBAC11

total BAC vector were apparent in four out of seven clones.

Identification of ASGR-linked BAC clones

Both BAC libraries were screened with six [^{32} P]-labeled ASGR-linked SCAR probes. However, only five out of six SCARs were used on both libraries. SCAR A14M was utilized as a probe onto the polyhaploid library but not on the apomictic buffelgrass in which this molecular marker is absent (unpublished results). C16, an ASGR-linked RFLP marker in buffelgrass, could not be mapped in an F_1 (*P. glaucum* × *P.squamulatum*) population segregating for mode of reproduction (unpublished results), thus it was not used to screen the polyhaploid library. The SCARs were able to identify 58 and 83 weakly to strongly cross-hybridizing clones in polyhaploid and buffelgrass libraries, respectively (Table 2). All BAC clones were characterized further by screening for the presence of the respective marker shown to be linked with apospory.

In the case of the polyhaploid, all six markers had been mapped in the total F_1 population as PCR products, therefore, PCR alone was initially used to assign clones to the ASGR. Out of 58 cross-hybridizing clones from the polyhaploid library, 25 (43%) showed amplification with the respective SCAR primers (Table 2). For Q8M, however, only 5 out of 12 clones showed PCR amplification of the SCAR marker even though Q8M was previously shown by RFLP analysis to be hemizygous; i.e., to hybridize at high stringency only to apomictic F_1 s (3 *Dra*I fragments) and not to any fragments in sexual F_1 s (Ozias-Akins et al. 1998). This prior result suggested that there might be RFLP markers that did not correspond to PCR-amplified SCAR markers but were nevertheless linked with apospory. By RFLP analysis of BACs, we were able to assign three additional polyhaploid, Q8M-hybridizing clones to the ASGR based on their content of an apospory-linked, but PCR-negative RFLP fragment.

In buffelgrass, only two types of BAC clones (UGT197, Q8M) were further characterized with SCAR primers since the remaining SCAR markers (A10H, C4, C16, O7M) were not apomict-specific and had been previously mapped as RFLPs (Roche et al. 1999). BACs isolated with these latter four markers required an analysis of RFLP content in which the polymorphic RFLP fragments previously mapped were used to determine which BAC clones could be assigned to the ASGR. For example, a total of ten BAC clones from the buffelgrass library were isolated with the SCAR C4 probe; however, only two of these hybridized to the same restriction fragments that cosegregated with the trait of apospory in buffelgrass (Fig. 3). The larger of the two cosegregating RFLP fragments was also present in *P. squamulatum*, the polyhaploid line and the apomictic F_1 (*P. glaucum* × *P. squamulatum*) 290-124, but not in the sexual F_1 290-105 (Fig. 3). Out of 83 buffelgrass clones, 28 (33%) were assigned to the ASGR based on their content of mapped markers. Similar to what we observed in the polyhaploid with Q8M BACs, one group of BACs from buffelgrass was not PCR-positive but could be assigned to the ASGR based on its content of one of two RFLP fragments cosegregating with apospory. In both species, apospory-linked RFLPs for Q8M could be separated into distinct BAC clones, one of which was SCAR PCR-positive and one of which was SCAR PCR-negative.

Table 2 Screening of BAC libraries from two apomictic grasses with probes mapped to the apospory-specific genomic region (ASGR). In all cases labeled probes were generated by PCR using [^{32} P]-dCTP, SCAR primers, and respective SCAR plasmid as template DNA. Analysis and picking of positively hybridizing clones were done following two washes of 30 min each at 65 °C in 0.5× and 0.1× SSPE plus 1% SDS, respectively (*N/A* not available)

Polyhaploid BAC library				Buffelgrass BAC Library		
ASGR-specific marker	Total BAC clones	ASGR PCR-positive	ASGR RPLF-positive	Total BAC clones	ASGR PCR-positive	ASGR RFLP-positive
A10H	5	2	–	12	–	1
A14M	3	3	–	N/A	N/A	N/A
C4	10	1	–	10	–	2
C16	N/A	N/A	N/A	17	–	0
O7M	19	6	–	22	–	7
Q8M	12	5	3	15	5	6
UGT197	9	8	–	7	7	N/A
Total	58	25	3	83	12	16

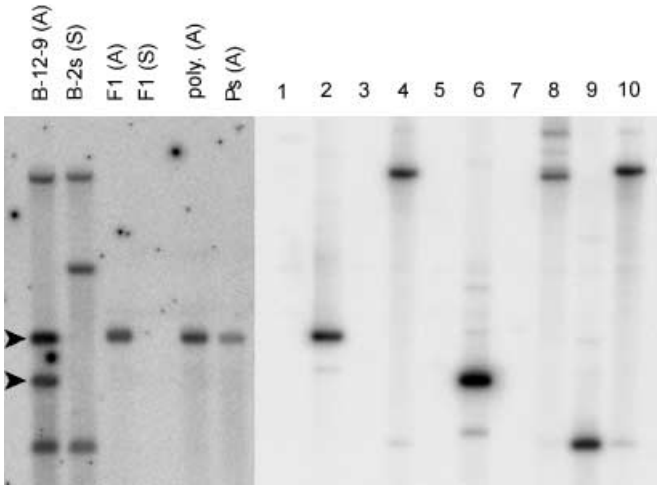

Fig. 3 Analysis of the RFLP content of C4-BAC clones relative to C4-hybridizing restriction fragments in genomic DNA. Southern-hybridization with [^{32} P]-labeled C4 SCAR to *Dra*I-cut genomic DNA of B-12-9 [apomictic (*A*)], B-2s [sexual (*S*)], an apomictic and sexual F_1 (290-124 and 290-105, respectively), polyhaploid (*poly*), *P. squamulatum* (*Ps*) and ten *Dra*I-cut C4 BAC clones (*lanes 1–10*) isolated from the buffelgrass library. Aliquots of 12.5 µg of total genomic DNA and 0.5 µg of each BAC clone were used per lane. Two polymorphic DNA fragments (*arrowheads*) linked to apospory in buffelgrass are found in the BAC clones of *lanes 2* and *6*

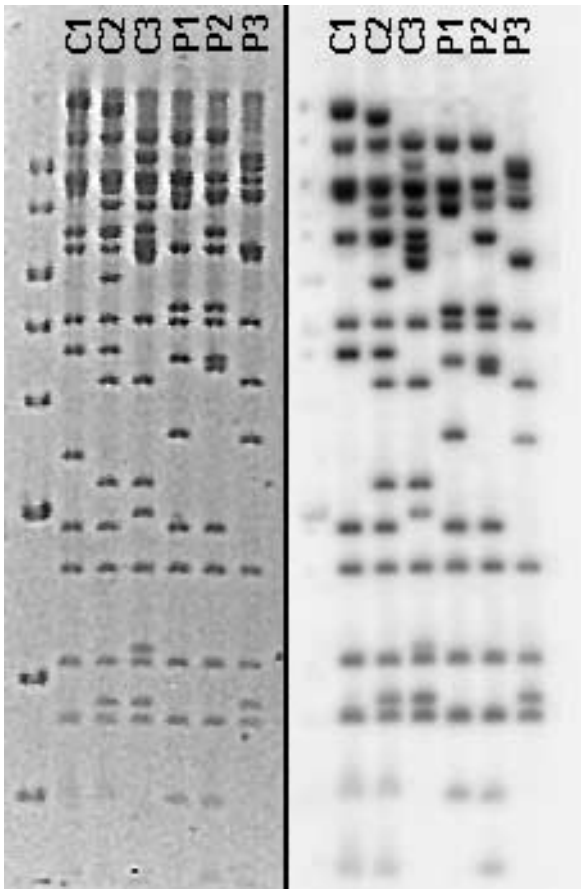

Fig. 4 Southern analysis of RFLP and homology conservation of Q8M PCR-negative BAC clones from buffelgrass and polyhaploid libraries. Plasmid extracts from six Q8M PCR-negative BAC clones from buffelgrass and polyhaploid libraries (prefixed *C* and *P*, respectively) were digested with *Eco*RI and separated by regular agarose gel electrophoresis (*left*). Southern analysis was carried out with a [^{32} P]-labeled C2 BAC (*right*). The first lane contains a l-kb ladder (NEB)

Polyhaploid and buffelgrass clones from Table 2 were grouped into SCAR-specific pools and further tested with SCAR markers that could not be used in filter hybridization due to repetitive elements within the SCAR. Six SCARs (P16R, R13, U12H, V4, W10M, X18R) (Ozias-Akins et al. 1998) were used to screen the polyhaploid pools, while four SCARs (P16R, U12H, V4, X18R) were screened against the buffelgrass pools since only these four had been mapped to the ASGR in buffelgrass (Roche et al. 1999). No amplification of these SCARs was observed on the BAC-clone pools (data not shown). From this initial screening in both species, no two SCARs were found in any one BAC clone, which suggests that the ASGR is likely to be larger than 1 Mbp in size.

Fingerprint analysis of BAC clones

In order to determine relationships between clones identified with various probes, a subset of ASGR-linked and unlinked clones were fingerprinted. Twelve contigs were assembled with seven clones remaining singletons. The contigs contained from two to six clones each. No contig assembled contained BAC clones from different SCAR markers, indicating that there was no substantial overlap between clones from different markers. The nine clones not shown to be linked with the ASGR fell into two contigs with one singleton. The separation of ASGR-linked and-unlinked clones verified that the conditions for contig assembly were strict enough to limit false associations of clones. A single contig was created for the three polyhaploid A14M clones. The seven polyhaploid UGT197 clones created two contigs of two clones each and three singletons. The seven buffelgrass UGT197 clones created two contigs of three and four clones. The six ASGR-linked polyhaploid O7M clones created two contigs of two and three clones and one singleton. Interestingly, the Q8M clones from the two libraries were contained within three contigs with one singleton. Six Q8M PCR-negative clones (three from each species) were grouped together in the same contig, suggesting that this region is highly conserved between the two species. These BAC clones were digested with *Eco*RI and probed with total labeled C2 BAC, which demonstrated that most *Eco*RI sites and homology were conserved between the two species (Fig. 4). The other two Q8M contigs contained the PCR-positive clones from the two libraries. Again, one of these contigs contained clones from both libraries.

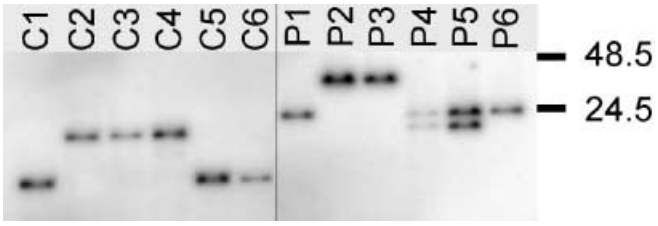

Fig. 5 Southern analysis of UGT197 PCR-positive BAC clones from buffelgrass and polyhaploid libraries. Plasmid extracts from six UGT197 PCR-positive BAC clones from each of the buffelgrass and polyhaploid libraries (prefixed *C* and *P*, respectively) were digested with *Not*I and separated by pulsed-field electrophoresis. Southern analysis was carried out with a [^{32} P]-labeled UGT197 SCAR. Two distinct classes of BACs were recovered from the buffelgrass library and three classes from the polyhaploid library. Sizes, in kilobases are indicated on the *right-hand side*

Duplication of ASGR markers in polyhaploid and buffelgrass

The identification of multiple contigs for individual SCAR markers was verified for the UGT197 clones through the use of RFLP data. Six buffelgrass and six polyhaploid ASGR-linked clones were digested with *Not*I and probed with the SCAR marker. Distinct *Not*I patterns were identified for both species (Fig. 5). None of the SCAR-hybridizing *Not*I fragments represented an insert-vector junction-fragment; therefore, the different size classes could be explained by linked duplications. This result demonstrates that there are at least two distinct regions of DNA in buffelgrass that correspond to the two FPC contigs and at least two, maybe more, distinct regions for the polyhaploid. Analysis with FPC software allowed the positioning of P2 and P3 within the same contig. P1 was not fingerprinted. Upon FPC analysis, P4 and P5 were not placed in the same contig, although their *Not*I restriction patterns were similar.

Discussion

We constructed BAC libraries from two grass species that reproduce by apospory. Both of these libraries have a low (0.3–1.2%) representation of organelle DNA among the clones. This low frequency of c1ones with cpDNA and mtDNA may be attributed to the use of nuclei as the megabase DNA source rather than protoplasts and also to the incorporation of 0.5% triton X-100 in successive steps throughout the preparation of nuclei. Usually fewer than 2% of the clones resulting from nuclei preparations contain cpDNA (Wang et al. 1995; Marek and Shoemaker 1997; Tomkins et al. 1999a, b; Nam et al. 1999; Budiman et al. 2000). In the construction of the polyhaploid BAC library we encountered high levels of false-positive clones found to have no inserts upon plasmid isolation and analysis. In the study reported here, we showed that partial degradation of the BAC vector was responsible for the “false-positive” nature of the transformed cells. The occurrence of false-positive clones has been previously attributed to the storage conditions of the dephosphorylated vector (Danesh et al. 1998) or to electroporation conditions (Lin et al. 1999). Since we used the same batch of frozen vector for successful and unsuccessful ligations and kept electroporation conditions constant across experiments, it is likely that additional factors played a role in the generation of false-positive clones. At 3%, the percentage of false-positive clones in the buffelgrass library was similar to that reported in soybean (Tomkins et al. 1999a), sugarcane (Tomkins et al. 1999b), and tomato (Budiman et al. 2000) libraries. The 12% false-positive clones in the polyhaploid library fell in the range of the 7–16% reported for rice (Wang et al. 1995), potato (Song et al. 2000), and *Medicago truncatula* (Nam et al. 1999). The BAC inserts from both buffelgrass and polyhaploid libraries typically produced more than one *Not*I restriction fragment, which is consistent with previous reports of a higher frequency of *Not*I sites in GC-rich grasses (Woo et al. 1994; Wang et al. 1995; Moullet et al. 1999; Tomkins et al. 1999b) compared with most dicots (Frijters et al. 1997; Tomkins et al. 1999a; Budiman et al. 2000).

Over 50 BAC clones that could be assigned to the ASGR were isolated from the two libraries after hybridization of the high-density filters with six low-copy SCAR clones. BAC clones were assigned to the ASGR in the polyhaploid library (25 or 5.0 per probe from an approximately 4× genome coverage after excluding the A14M clones that had no counterpart from buffelgrass) and in the buffelgrass library (28 or 5.6 per probe from an approximately 5× genome coverage). The representation of a probe in our libraries did not differ substantially from the two to eight clones per probe recovered from two soybean libraries, each with 4.7 haploid genome equivalents (Meksem et al. 2000). The larger total number of clones from buffelgrass (83 vs. 58 from polyhaploid) could be due to the slightly greater genome coverage of the library, but also to the larger number of unmapped, multiplex RFLP fragments detected in genomic DNA that could represent allelic, linked, or unlinked sequences (Ozias-Akins et al. 1998; Roche et al. 1999).

Here we provided examples (for SCARs – C4, UGT197, Q8M) of the feasibility of using BAC clones to study the microcolinearity of the ASGR in both species. BAC clones conserved partially or totally between the two species may harbor the gene(s) of interest for this mode of reproduction. However, at the onset of library construction, and based on our molecular marker mapping in both species, we did not expect to find as many classes of BAC clones and separate DNA contigs for each SCAR. It seems that the ASGR in both species comprises several duplicated regions. We have previously documented that the ASGR is partially hemizygous in nature and lacks genetic recombination (Ozias-Akins et al. 1998). Furthermore, its nucleotide divergence for non-coding nuclear DNA regions between the two species is similar to that observed between non-coding regions of the chloroplast genome in the same two species (Roche et al. 1999). Some of the ASGR-linked markers have

also been found in other *Pennisetum* species (Lubbers et al. 1994 and unpublished). It is possible that the ASGR was organized prior to speciation within the *Pennisetum*/*Cenchrus* complex. Considering that apomixis may significantly increase the fitness of progenies issued from interspecific crosses (Harlan and de Wet 1963), the ASGR may have moved laterally through different grasses of sub-Saharan Africa, the geographical region from which *Pennisetum squamulatum* and *Cenchrus ciliaris* originated.

To determine if any of the remaining mapped SCAR markers could be located within any of the isolated BAC clones, each pool of BAC clones was tested by PCR with the other SCARs. None of these PCR analyses was positive, indicating that none of the isolated clones contained more than one SCAR marker. Before these analyses we considered the possibility that the ASGR may be a relatively small, non-recombining genomic region. In this case, a bulked-segregant analysis in extremely heterozygous F_1 progenies may have efficiently saturated the region with molecular markers (Ozias-Akins et al. 1998). However, our current findings in the polyhaploid line indicate that at least 28 ASGR-assigned BAC clones do not include more than one SCAR marker. The amount of overlap among these 31 clones is not finalized as the end-clone analysis is in progress and end clones are being used for walking. However, with an average insert six of 82 kb it is likely that the ASGR, as previously defined with 12 independent SCARs (Ozias-Akins et al. 1998), may be at least several hundred kilobase pairs in size.

Physical mapping of the ASGR in buffelgrass and *P. squamulatum* will be expanded by application of the BAC resource using a combination of DNA contig assembly and fluorescence in situ hybridization (FISH). The latter technique has proved to be essential for resolving physical distances in complex organisms where gaps in DNA contigs frequently occur (Jackson et al. 1998) or may be obscured by duplications (Stupar et al. 2001). The two BAC libraries we constructed will be invaluable tools to decipher the organization and eventually the gene content of the ASGR in both species.

Acknowledgements We are grateful for the technical assistance of Anne Bell, Evelyn Perry, Jacolyn Merriman (USDA-ARS), and Sue Dove at the Coastal Plain Experiment Station, and Michael Atkins, John Bishop, Barbara Blackmon, and Scheen Thurmond at the Clemson University Genomics Institute. We acknowledge support of D. Roche by Pioneer Hybrid International and Limagrain SA through a cooperative research and development agreement, of JA Conner by a DOE-EnergyBiosciences research fellowship, as well as comprehensive support of our work by the USDANRI Plant Genome Program, award nos. 93-37304-9363 and 99-35300-7691. Experiments have been conducted in compliance with current US laws.

References

Asker S, Jerling L (1992) Apomixis in plants. CRC Press, Boca Raton

Bennett MD, Leitch IJ (1997) Nuclear DNA amounts in angiosperms – 583 new estimates. Ann Bot 80:169–196

Budiman MA, Mao L, Wood TC, Wing RA (2000) A deep-coverage tomato BAC library and prospects toward development of an STC framework for genome sequencing. Genome Res 10:129–136

Burke DT, Carle GF, Olson M (1987) Cloning of large fragments of exogenous DNA into yeasts by means of artificial chromosome vectors. Science 236:806–812

Cai L, Taylor JF, Wing RA, Gallagher DS, Woo SS, Davis SK (1995) Construction and characterization of a bovine bacterial artificial chromosome library. Genomics 29:413–425

Danesh D, Penuela S, Mudge J, Denny RL, Nordstrom H, Martinez JP, Young ND (1998) A bacterial artificial chromosome library for soybean and identification of clones near a major cyst nematode resistance gene. Theor Appl Genet 96:96–202

Demesure B, Sodzi N, Petit RJ (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol Ecol 4: 129–131

Dujardin M, Hanna WW (1986) An apomictic polyhaploid obtained from a pearl millet × *Pennisetum squamulatum* apomictic interspecific hybrid. Theor Appl Genet 72:33–36

Frijters ACJ, Zhang Z, van Damme M, Wang GL, Ronald PC, Michelmore RW (1997) Construction of a bacterial artificial chromosome library containing large *Eco*RI and *Hin*dIII genomic fragments of lettuce. Theor Appl Genet 94:390–399

Gustine DL, Sherwood RT, Huff DR (1997) Apospory-linked molecular markers in buffelgrass. Crop Sci 37:947–951

Hanna WW (1995) Use of apomixis in cultivar development. Adv Agron 54:333–350

Harlan JR, de Wet JMJ (1963) Role of apomixis in the evolution of the *Bothriochloa-Dichanthium* complex. Crop Sci 3: 314–316

Jackson SA, Wang ML, Goodman HM, Jiang J (1998) Application of fiber-FISH in physical mapping of *Arabidopsis thaliana.* Genome 41:566–572

Lin Y-R, Zhu L, Ren S, Yang J, Schertz KF, Paterson AH (1999) A *Sorghum propinquum* BAC library, suitable for cloning genes associated with loss-of-function mutations during crop domestication. Mol Breed 5:511–520

Lubbers EL, Arthur L, Hanna WW, Ozias-Akins P (1994) Molecular markers shared by diverse apomictic *Pennisetum* species. Theor Appl Genet 89:636–642

Marek LF, Shoemaker RC (1997) BAC contig development by fingerprint analysis in soybean. Genome 40:420–427

Marie D, Brown SC (1993) A cytometric exercise in plant DNA histograms, with 2C values for 70 species. Biol Cell 78:41–51

Martel E, De Nay D, Siljak-Yakovlev S, Brown S, Sarr A (1997) Genome size variation and basic chromosome number in pearl millet and fourteen related *Pennisetum* species. J Hered 88: 139–143

Meksem K, Zobrist K, Ruben E, Hyten D, Quanzhou T, Zhang H-B, Lightfoot DA (2000) Two large-insert soybean genomic libraries constructed in a binary vector: applications in chromosome walking and genome wide physical mapping. Theor Appl Genet 101:747–755

Moullet O, Zhang H-B, Lagudah ES (1999) Construction and characterization of a large DNA insert library from the D genome of wheat. Theor Appl Genet 99:305–313

Nam Y-W, Penmetsa RV, Endre G, Uribe P, Kim D, Cook DR (1999) Construction of a bacterial artificial chromosome library of *Medicago truncatula* and identification of clones containing ethylene-response genes. Theor Appl Genet 98:638–646

Nogler GA (1984) Gametophytic apomixis. In: Johri BM (ed) Embryology of angiosperms. Springer, Berlin Heidelberg New York, pp 475–518

Otto FJ (1994) High-resolution analysis of nuclear DNA employing the fluorochrome DAPI. Methods Cell Biol 41: 211–217

Ozias-Akins P, Pring DR, Vasil IK (1987) Rearrangements in the mitochondrial genome of hybrid cell lines of *Pennisetum americanum* (L.) K. Schum. + *Panicum maximum* Jacq. Theor Appl Genet 74:15–20

Ozias-Akins P, Roche D, Hanna WW (1998) Tight clustering and hemizygosity of apomixis-linked markers in *Pennisetum squamulatum* implies genetic control of apospory by a divergent locus that may have no allelic form in sexual genotypes. Proc Natl Acad Sci USA 95:5127–5132

Roche D, Cong P, Chen Z, Hanna WW, Gustine DL, Sherwood RT, Ozias-Akins P (1999) An apospory-specific genomic region is conserved between buffelgrass (*Cenchrus ciliaris* L.) and *Pennisetum squamulatum* Fresen. Plant J 19:203–208

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sherwood RT, Berg CC, Young BA (1994) Inheritance of apospory in buffelgrass. Crop Sci 36:1490–1494

Shizuya H, Birren B, Kim U, Mancino V, Slepak T, Tachiiri U, Simon M (1992) Cloning and stable maintenance of 300-kilobasepair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc Natl Acad Sci USA 89:8794–8797

Smith RL, Chowdhury MKU, Pring DR (1987) Mitochondrial DNA rearrangements in *Pennisetum* associated with reversion from cytoplasmic male sterility to fertility. Plant Mol Biol 9:277–286

Song J, Dong F, Jiang J (2000) Construction of a bacterial artificial chromosome (BAC) library for potato molecular cytogenetics research. Genome 43:199–204

Stupar RM, Lilly JW, Town CD, Cheng Z, Kaul S, Buell CR, Jiang J (2001) Complex mtDNA constitutes an approximate 620-kb insertion on *Arabidopsis thaliana* chromosome 2: Implication of potential sequencing errors caused by large-unit repeats. Proc Natl Acad Sci USA 98:5099–5103

Tomkins JP, Mahalingam R, Smith H, Goicoechea JL, Knap HT, Wing RA (1999a) A bacterial artificial chromosome library for soybean PI 437654 and identification of clones associated with cyst nematode resistance. Plant Mol Biol 41:25–32

Tomkins JP, Yu Y, Miller-Smith H, Frisch DA, Woo SS, Wing RA (1999b) A bacterial artificial chromosome library for sugarcane. Theor Appl Genet 99:419–424

Wang G-L, Holsten TE, Song W-Y, Wang H-P, Ronald PC (1995) Construction of a rice bacterial artificial chromosome library and identification of clones linked to the *Xa-21* disease resistance locus. Plant J 7:525–533

Woo S-S, Jiang J, Gill BS, Paterson AH, Wing RA (1994) Construction and characterization of a bacterial artificial library of *Sorghum bicolor*. Nucleic Acids Res 22:4922–4931

Zhang HB, Zhao XP, Ding XL, Paterson AH, Wing RA (1995) Preparation of megabase-size DNA from plant nuclei. Plant J 7:175–184