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Protocol

Isolation of Megabase-Size DNA from Sorghum and Applications for Physical Mapping and Bacterial and Yeast Artificial Chromosome Library Construction

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Abstract: A method was developed for the isolation of megabase-size DNA from *Sorghum bicolor*. Sorghum protoplasts were isolated from young leaf tissue, embedded in an agarose matrix as microbeads or plugs, followed by cell lysis and protein degradation. The DNA prepared by this method was larger than 1 Mb in size and readily digestible with restriction enzymes. The DNA was shown to be suitable for physical mapping, and was successfully used for the construction of BAC and YAC libraries.

The recent developments of PFGE technology (Schwartz and Cantor, 1984; Chu et al., 1986) and cloning systems for large DNA fragments, such as YACs (Burke et al., 1987) and BACs (Shizuya et al., 1992), has led to valuable information about complex genomes of

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Abbreviations: BAC, bacterial artificial chromosome; CHEF, clamped homogeneous electric field; CIAP, calf intestine alkaline phosphatase; LMP, low melting point; LMW, low molecular weight; PFGE, pulsed-field gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SPD, spermidine; YAC, yeast artificial chromosome.

higher organisms and to the isolation of genes known only by map position and phenotype. A prerequisite for such investigations is DNA of high molecular weight (megabase-size) and high quality. Because plant cells have rigid cell walls, it is more demanding to isolate such DNA from plants than from mammalian cells. The most common methods for plant megabase-size DNA isolation include isolation of protoplasts or nuclei, embedding of protoplasts or nuclei in an agarose matrix (agarose plugs or microbeads), and cell lysis and protein degradation.

Recently, techniques for the isolation of megabase-size DNA have been developed for a number of plant species (Ganal and Tanksley, 1989; Daelen et al., 1989; Cheung and Gale, 1990; Sobral et al., 1990; Honeycutt et al., 1992; Hatano et al., 1992; Guidet and Langridge, 1992; Wing et al., 1993; Zhang et al., 1995). The DNA isolated by these techniques has been successfully applied to large-scale physical mapping of specific chromosome regions in plant genomes (Ganal et al., 1989; Cheung et al., 1991; Bancroft et al., 1992; Wing et al., 1994; Zhang et al., 1994), construction of YAC libraries (Ward and Jen, 1990; Grill and Somerville, 1991; Dunford and Rogner, 1991; Martin et al., 1992; Edwards et al., 1992; Umehara et al., 1992; Eyers et al., 1992), and map-based (positional) cloning of economically important genes (Arondel et al., 1992; Leyser et al., 1993; Martin et al., 1993). A method to isolate megabase-size DNA from sorghum (*Sorghum bicolor*), however, has not been reported.

Sorghum has a relatively small haploid genome of approximately 750 Mb (Arumuganathan and Earle, 1991), and at least six RFLP linkage maps for sorghum have been developed (Hulbert et al., 1990; Binelli et al., 1992; Whitkus et al., 1992; Berhan et al., 1993; Chittenden et al., 1994; Xu et al., 1994; Pereira et al., 1994). Sorghum is closely related to maize (Hulbert et al., 1990; Binelli et al., 1992; Whitkus et al., 1992; Chittenden et al., 1994), both of which are important grain crops worldwide. Recent studies revealed the similarity and conservation of many genomic regions of cereal crop plants (Ahn et al., 1993; Ahn and Tanksley, 1993). Therefore analysis of the physical structure of the sorghum genome and cloning of large DNA fragments would provide further understanding of sorghum and other cereal genomes. To facilitate such investigations, it is necessary to have a feasible method to isolate and manipulate megabase-size DNA from sorghum.

In this paper we report a technique for the isolation of megabase-size DNA from protoplasts of *Sorghum bicolor* (L.) Moench, and the use of such DNA for physical mapping, and YAC and BAC cloning.

Materials and Methods

Plant materials

Sorghum bicolor cv. BTx623 was grown in flat trays in a growth chamber with cool white fluorescent lights. When the plants were 15-18 days old, young leaves of 8-10 cm in length were harvested, and the tip and basal portions of each leaf were removed.

Chemicals and equipment

protoplast buffer:[1] 0.5 M sorbitol, 10 mM $CaCl_2 2H_2O$, pH 5.5 (Earle et al., 1978)
protoplast isolation buffer: 2% cellulysin (CALBIOCHEM, USA) in protoplast buffer
SCE::[1] M sorbitol, 0.1 M sodium citrate, 60 mM EDTA, pH 7.0
LMP agarose (GIBCO BRL, USA)
Light mineral oil (Mallinckrodt #6358)
ESP: 0.5 M EDTA, pH 9.3,[1] 1 % Na lauryl sarcosine,[1] 0.1 mg/mL proteinase K (1 mg/mL for plugs)
TE:[1] 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
CIAP (BRL, USA)
β-agarase (New England Biolabs, USA)
carborundum,[2] 320 grit (Fisher, USA)
paint brush: 2-cm-wide water color brush
PMSF[3] (Sigma, USA): 100 mM in isopropanol
30- and 80-µm mesh (Spectrum, USA)

Notes

1. Autoclaved
2. Carborundum is a potential carcinogen; therefore, protective clothing should be worn at all times (e.g. gloves, mask, goggles, and lab coat).
3. PMSF is a highly toxic acetylcholinesterase inhibitor. To avoid possible lab contamination, we purchase small 250-mg aliquots and then add 14.35 mL of isopropanol to the entire bottle to make a final concentration of 100 mM.

Protocol

Isolation of protoplasts

- Sprinkle the underside of each leaf piece with 320 grit carborundum and brush 50 times in each direction with a 2 cm-wide water color brush in a fume-hood.
- Wash the carborundum-treated leaves five times thoroughly with distilled water in a beaker.

- Transfer 2 to 2.5 g of the leaves underside down to 32 mL of protoplast isolation buffer in a large petri dish (150x15 mm). Incubate the leaves in the dish on a gyratory shaker (50 rpm) at room temperature in darkness until the leaves begin to break up (1-2 hours), and then add an equal volume (32 mL) of protoplast buffer without cellulysin (Earle et al., 1978) to the incubating solution.
- After four and a half hours from the initial incubation, check the release of protoplasts under a microscope. Stop the incubation if most of the protoplasts are released from the leaf tissues.
- Filter the solution containing protoplasts through a 80-μm mesh and then a 30-μm mesh.
- Pellet the protoplasts for 10 min at 35xg at room temperature using a swinging bucket rotor with round bottom Oak Ridge tubes; discard the supernatant fluid, combine, and resuspend the pellets in 40 mL of fresh protoplast buffer. Repeat this step once, and finally combine the protoplasts in a single Oak Ridge tube and pellet as above.
- Count the number of protoplasts under the microscope using a hemacytometer during the third centrifugation step.

Notes

1. We failed to isolate protoplasts from plants grown in a greenhouse. We usually grew two flat trays of seedlings and harvested 40 g of leaf tissues, which yielded approximately $1.0\text{-}1.5 \times 10^7$ protoplasts per g of fresh leaf tissue.
2. The duration of brushing with carborundum can be increased or decreased according to the stiffness of each leaf.

Embedding protoplasts in an agarose matrix

Microbead preparation. Microbeads are prepared as described previously by Wing et al. (1993) with slight modifications. The procedures briefly are:

- Resuspend 2.0×10^8 protoplasts (for BAC cloning: 6.25×10^8) in 5 mL of SCE and pour into a 300-mL flask prewarmed at 45°C.
- Prewarm the protoplast solution to 45°C, and add an equal volume of 1% LMP agarose in SCE kept at the same temperature. Quickly mix the solution.
- Immediately add 20 mL of light mineral oil kept at 45°C, shake the contents of the flask vigorously for 3-5 seconds, and then pour the mixture into a 500 mL beaker containing 150 mL of ice-cold SCE buffer while stirring with a magnetic stir bar.
- Let the resulting emulsion stir for 5 min in an ice bath.
- Pellet microbeads at 500xg for 10-15 min.
- Pipette off most of the mineral oil except at the interface which contains a significant amount of microbeads. Combine the interface and supernatant fluid, and pellet as above.

Agarose plug preparation

- Resuspend $1.0x10^8$ protoplasts in 5 mL of protoplast buffer and warm protoplasts to 45°C. Gently mix the protoplasts with 5 mL of 1% LMP agarose in protoplast buffer kept at 45°C.
- Dispense 100-µL aliquots of the mixture into a prechilled mold and incubate on ice for 5 min to solidify the agarose plugs.

Notes

1. The amount of sorghum DNA per 100-µL plug was approximately 1.6 µg, based on a genome size of 1.6 pg per diploid nucleus (Arumuganathan and Earle, 1991), since $1.0x10^8$ protoplasts were embedded in 10 mL of agarose plugs.
2. The approximate amount of sorghum DNA per 100 µL of microbeads was 3.2 µg because $2.0x10^8$ protoplasts were embedded in 10 mL of agarose microbeads.
3. High DNA concentration microbeads for BAC cloning were prepared by embedding $6.25x10^8$ protoplasts in 10 mL of agarose microbeads, which had the approximate concentration of 10 µg of DNA per 100 µL of microbeads.

Cell lysis and protein degradation

- Combine all of the plugs or pelleted microbeads prepared from 40 g of leaf tissue into two 50-mL Falcon tubes, and incubate in 100 mL of ESP at 50°C with gentle shaking for 24 hours.
- Change the ESP solution and incubate the plugs or microbeads for an additional 12–24 hours.
- Before digesting the DNA with restriction enzymes, wash the agarose microbeads or plugs six times with TE on ice, one hour each. The first three washes include 0.1 mM PMSF to inactivate the proteinase K.

Restriction enzyme digestion for physical mapping

- Set up each digestion reaction using 100 µL of microbeads (approx. 3.2 µg DNA), 1/10 volume of 10X restriction enzyme buffer, 1/10 volume of 40 mM SPD, and 30-40 units of restriction enzyme in a final volume of 150 µL.
- Incubate the reaction overnight at the recommended temperature and stop the reaction by addition of 15 µL of 0.5 M EDTA, pH 8.0. Analyze the digests by PFGE.

Partial digestion for BAC and YAC cloning

Microbeads (high DNA concentration) for BAC cloning

- Pre-incubate 50 µL of microbeads containing approximately 5 µg of sorghum megabase DNA with 1X *Hind* III reaction buffer (6 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, pH 7.5), 100 µg/mL BSA, and 4 mM SPD in a final volume of 100 µL on ice for 20 min.

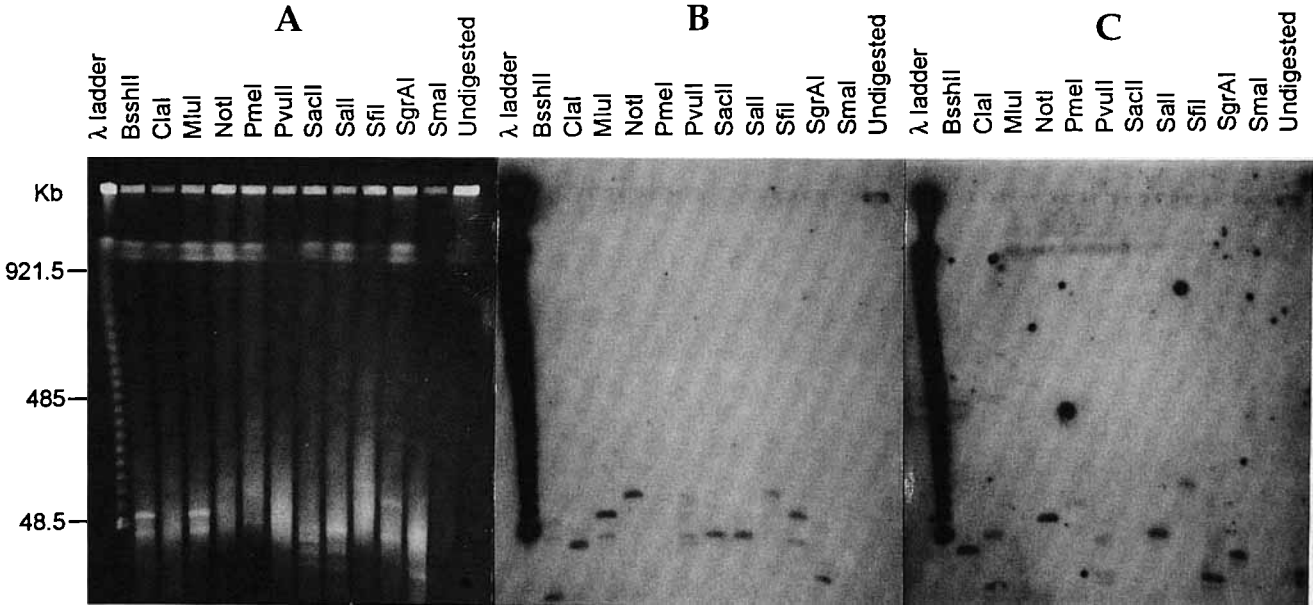

Fig. 1. Analysis of DNA from *S. bicolor* by PFGE and Southern blots. A: Megabase-size DNA from *S. bicolor* analyzed by PFGE. The DNA was digested with restriction enzymes as indicated: *Bss* HII, *Cla* I, ... or *undigested*. **B** and **C**: Southern hybridization of the PFGE gel of sorghum DNA with single-copy probes tightly linked on a RFLP linkage map (Chittenden et al. 1994). Note that pSB639 in **B** and pSB562 in **C** both hybridized to the 50-kb *Sal*I fragments. The DNA was separated by PFGE in 1% agarose gel, 0.5X TBE, 14°C, and 150 Volts for 40 h with the MJ Research (USA) Programmable Power Inverter: A= 100 s; B= -0.05 s; C= 100 s; D= -0.05 s; E= 175 cycles; F= -0.01 s; G= -0.01 s.

- Add 2 units of *Hind* III (Promega, USA) and incubate on ice for additional 20 min to allow the enzyme to diffuse into the beads.
- Incubate the reaction in a 37°C water bath for 5 min and stop the reaction by addition of 1/10 volume of 0.5 M EDTA, pH 8.0 on ice.

Plugs for YAC cloning

- Melt the plugs at 67°C for 10 min to release megabase-size DNA and digest the agarose with β-agarase as specified by manufacturer.
- Digest 3 sets of 90 µL of megabase-size DNA in liquid phase in a reaction cocktail containing 1X *Eco*RI reaction buffer (90 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.5) and 4 mM SPD in a final volume of 180 µL at 37°C for 20 min with 6.3 units, 18 units, and 54 units of *Eco*RI (Promega, USA).
- Combine the digests together for size selection using PFGE.

Results and Discussion

Physical mapping

To test the suitability of DNA prepared from *S. bicolor* protoplasts for physical mapping, the DNA was digested with a number of restriction enzymes and analyzed by PFGE (Fig. 1A). Most digests with these enzymes show an increase in fluorescence of DNA in the compression zone greater than 1 Mb in size as compared to the uncut control (*undigested*) indicating that the DNA is digestible with these restriction enzymes. Additionally, Fig. 1A. shows distinct digestion patterns in all of the lanes. The mean size ranges for the *Pvu*II and *Sma*I digests are approximately 150 kb whereas the remaining digests have ranges from 50 to 1000 kb in size. The digestion patterns suggest that these enzymes are useful for physical mapping of the sorghum genome in the 50- to 1000-kb range. The CHEF gel in Fig. 1A was Southern blotted according to Lee et al. (1991) and hybridized with two tightly linked sorghum RFLP probes, pSB562 and pSB639, less than 1 cM apart on linkage group J (Chittenden et al., 1994). Southern analysis revealed that whereas probe pSB639 (Fig. 1B) hybridized to specific bands in all the digested DNA lanes, probe pSB562 (Fig. 1C) hybridized to specific bands in all but the *Mlu*I and *Sac*II lanes. The later result for the *Mlu*I and *Sac*II digests may have occurred because the restriction fragments that could hybridize with pSB562 ran off the gel or hybridized to the DNA in the compression zone of the gel and thus could not be detected. The size range of hybridized fragments for these two probes is between 10 and 300 kb.

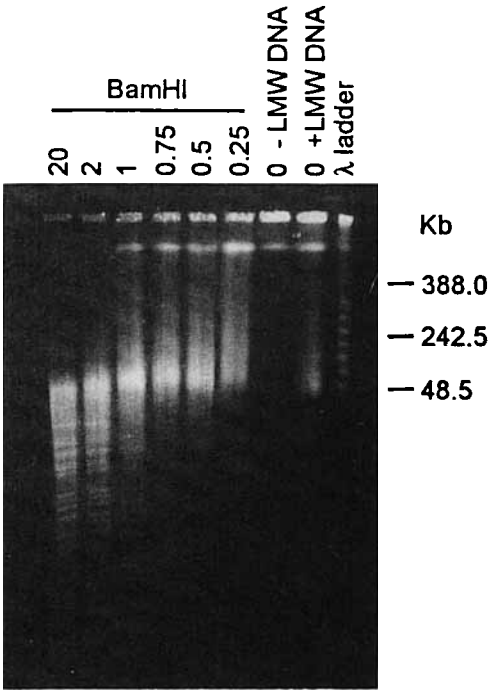

Fig. 2. Partial digestion of DNA from *S. bicolor* with *Bam* HI. Partially digested *S. bicolor* megabase-size DNA in agarose microbeads with *Bam* HI for 15 min at 37°C. Lanes 1–6 contain DNA digested with indiated units of *Bam* HI. Lanes marked *0 -LMW DNA* and *0 +LMW DNA* contain uncut DNA after and before pre-electrophoresis, respectively. The DNA was separated by PFGE on a 1% agarose gel in 0.5X TBE at 14°C, with 6.0 V / cm, 40-s pulse time for 18 h using the HEXCHEF 6000 (CBS-Scientific, USA) set with the MJ Research Programmable Power Inverter.

Only one 50-kb *SalI* band was common between both probes. This result suggests that both probes may share the same 50-kb fragment or hybridize to two comigrating 50-kb fragments. Nevertheless, these results clearly indicate the suitability of *S. bicolor* megabase-size DNA prepared by this method for physical mapping.

Partial digestion

The construction of large-insert BAC and YAC libraries requires megabase-size DNA that can be partially digested with restriction enzymes. Therefore, we tested susceptibility of this DNA to *Bam* HI partial digestions. To properly calibrate partial digestion conditions, low molecular weight (LMW) DNA was first removed from the agarose microbeads by PFGE at 6.0 V / cm in a 1% agarose gel, using a pulse time of 40 s for 18 hours. Under these conditions most LMW DNA less than 500 kb was removed; the majority of high molecular weight (HMW) DNA, however, remained in the agarose microbeads (Fig. 2, *0 -LMW DNA*). The *0 -LMW DNA* and *0 +LMW DNA* lanes in Fig. 2 show sorghum HMW

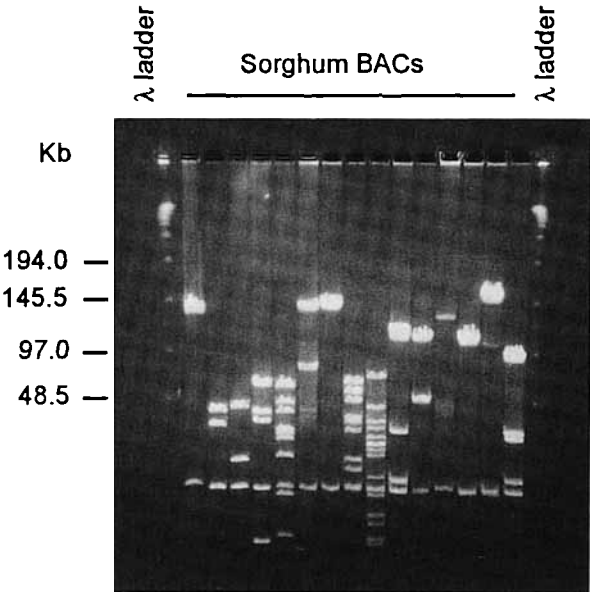

Fig. 3. Analysis of *S. bicolor* BAC clones by PFGE. Lanes between the λ ladders contain 15 randomly picked recombinant BAC clones prepared by alkaline lysis and digested with *Not*I. PFGE was performed with the CHEF Mapper (Bio-Rad, USA) with a linear pulse time ramping from 5 to 15 s under conditions; 6.0 V/cm, 11°C, for 17 h, 0.5X TBE, with 1% agarose gel.

DNA after and before pre-electrophoresis used to remove the LMW DNA, respectively. After pre-electrophoresis, equal amounts of sorghum DNA were digested with serially diluted *Bam* HI and then analyzed by PFGE. Fig. 2 shows that only a small amount of *Bam* HI (0.25-1.0 units) was sufficient to digest the DNA in 50 μL of agarose microbeads (approximately 1.6 μg) producing DNA fragments in the 100- to 500-kb range. When 20 units of *Bam* HI were used, the DNA was completely digested, giving a size range below 50 kb (Fig. 2, lane 1). These results demonstrate that the megabase-size DNA in agarose microbead form is sensitive to relatively small amounts of *Bam* HI, and should be suitable for the random partial digestions required for BAC and YAC cloning.

BAC and YAC Cloning

We tested the BAC and YAC cloning properties of sorghum megabase-size DNA prepared by this method. We constructed a partial sorghum YAC library of approximately 2,000 clones and a sorghum BAC library of 13,440 clones with an average insert size of 157 kb (Woo et al., 1994).

For BAC cloning, the procedure of Woo et al. (1994) was followed with partially digested *S. bicolor* megabase DNA in microbeads with *Hind* III and pBeloBAC11 provided by H. Shizuya and M. Simon (Caltech). Fig.

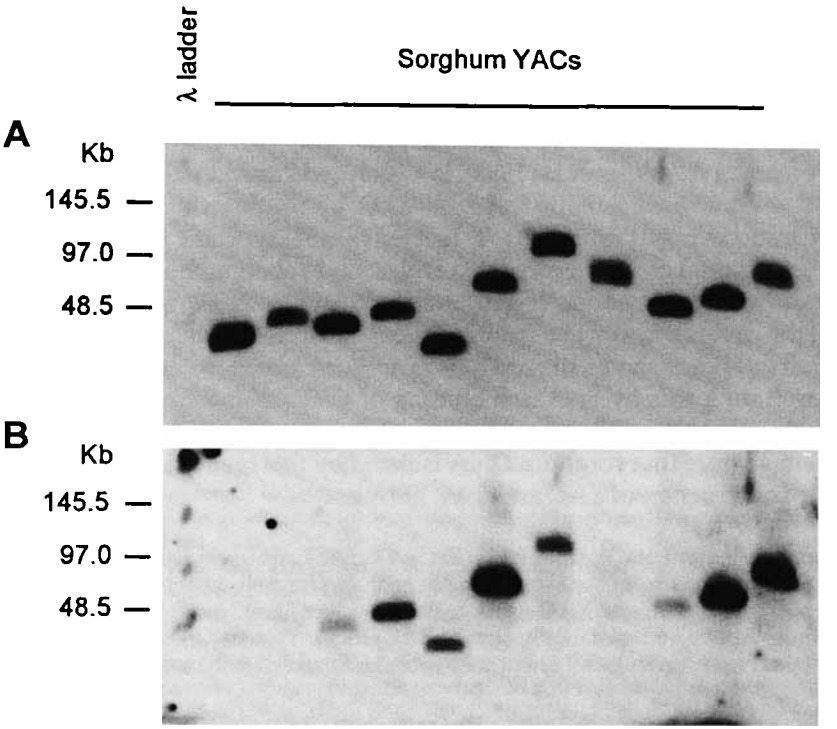

Fig. 4. Southern blots of sorghum YACs. Autoradiographs of Southern blot filters from CHEF gels of 11 independent sorghum YACs hybridized sequentially with **A**: pBR322 DNA, and **B**: total *S. bicolor* genomic DNA.

3 shows the analysis of 15 random BAC clones in a size range of 70 to 335 kb. The BAC DNA was isolated by alkaline lysis (Silhavy et al., 1984) from 5 mL overnight cultures in LB + 12.5 μg/mL chloramphenicol and digested with *Not*I to release the insert from the vector and resolved on a CHEF gel. The common band in all the lanes (7.4 kb) is from the vector, and the remaining bands are derived from *S. bicolor* DNA.

For YAC cloning, the DNA partially digested with *Eco*RI was combined and size selected by PFGE (Anand et al., 1989) using the CBS Scientific HEX-CHEF 6000 at 150 voltages, in a 1 % LMP agarose gel, with an 8-s pulse for 6 hours at 11°C running in 0.5X TBE buffer. A gel slice containing DNA greater than 100 kb in size and the well was excised from the gel, and melted at 67°C followed by digestion with β-agarase at 40°C

for one hour as specified by the manufacturer. After digestion the DNA was dialyzed against TE overnight. The size-selected DNA was ligated into pYAC4, previously digested with *Bam* HI and *Eco*RI and dephosphorylated with CIAP. *Saccharomyces cerevisiae* strain AB1380 was transformed with the ligated mixture as previously described (Burgess and Percival, 1987; Wing et al., 1993). We analyzed 22 randomly picked yeast transformants containing sorghum YACs by Southern blotting of CHEF gels and hybridization with ^{32}P-labeled pBR322 (specific for the pYAC4 vector), total *S. bicolor* genomic DNA, and tobacco chloroplast DNA sequentially. The sorghum YACs range from 40 to 210 kb in size, and one of the clones appears to contain chloroplast-derived sequences (data not shown). Fig. 4 shows the analysis of 11 yeast transformants containing sorghum YACs by Southern blotting of CHEF gels and hybridization with ^{32}P-labeled pBR322 and total *S. bicolor* genomic DNA. These results demonstrate that sorghum DNA isolated by this method is suitable for the construction of YAC and BAC libraries.

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