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Construction and characterization of a bacterial artificial chromosome library of *Sorghum bicolor*

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ABSTRACT

The construction of representative large insert DNA libraries is critical for the analysis of complex genomes. The predominant vector system for such work is the yeast artificial chromosome (YAC) system. Despite the success of YACs, many problems have been described including: chimerism, tedious steps in library construction and low yields of YAC insert DNA. Recently a new *E.coli* based system has been developed, the bacterial artificial chromosome (BAC) system, which offers many potential advantages over YACs. We tested the BAC system in plants by constructing an ordered 13 440 clone sorghum BAC library. The library has a combined average insert size, from single and double size selections, of 157 kb. Sorghum inserts of up to 315 kb were isolated and shown to be stable when grown for over 100 generations in liquid media. No chimeric clones were detected as determined by fluorescence *in situ* hybridization of ten BAC clones to metaphase and interphase *S.bicolor* nuclei. The library was screened with six sorghum probes and three maize probes and all but one sorghum probe hybridized to at least one BAC clone in the library. To facilitate chromosome walking with the BAC system, methods were developed to isolate the proximal ends of restriction fragments inserted into the BAC vector and used to isolate both the left and right ends of six randomly selected BAC clones. These results demonstrate that the *S.bicolor* BAC library will be useful for several physical mapping and map-based cloning applications not only in sorghum but other related cereal genomes, such as maize. Furthermore, we conclude that the BAC system is suitable for most large genome applications, is more 'user friendly' than the YAC system, and will likely lead to rapid progress in cloning biologically significant genes from plants.

INTRODUCTION

Physical mapping and chromosome walking in complex genomes requires large insert DNA libraries that can be rapidly constructed, easily screened and manipulated and are relatively free of chimerism. Most large insert DNA libraries are constructed in bacteriophage, cosmid and yeast artificial chromosome (YAC) cloning vectors. The insert size of bacteriophage and cosmid libraries are limited by phage particle size and have a maximum practical limit of up to 17 kb (lambda), 46 kb (cosmid) (1) and 100 kb (P1) (2). The primary advantage of bacteriophage libraries is that they are easy to generate and screen. YAC libraries, originally introduced in 1987 by Burke *et al.* (3), have revolutionized the analysis of complex genomes. The cloning capacity in YACs appears to be limitless and primarily depends on the size and integrity of the insert DNA. Many YAC libraries have been constructed for a variety of organisms (4–8) including plants (9–13) and have been invaluable for the assembly of large physical contigs (14–16) and the isolation of many important genes (reviewed in 17,18–20). The primary advantage of using YACs is the large insert size which is important for many chromosome walking applications. Despite the success of the YAC cloning system many problems have been found which include chimerism (8,21,22), and tedious steps in manipulation and isolation of YAC insert DNA. Furthermore, the construction of comprehensive YAC libraries from organisms with large genomes is technically demanding, especially for plants.

Recently two new *E.coli* based large DNA cloning systems have been developed—the bacterial artificial chromosome (BAC) system (23) and the P1-derived artificial chromosome (PAC) system (24). Shizuya *et al.* (1992) described the construction of a F factor derived vector system that was used to clone human DNA and found inserts as large as 300 kb (23). The PAC system combines a positive selection for recombinant clones with the BAC system by incorporating the SacB gene which is toxic to *E.coli* when plated on saccharose media (24). Both BAC and PAC vectors are maintained as single copy supercoiled plasmids in *E.coli* and exclude other BAC and PAC plasmids from replicating in the same host cell. Potential advantages of the BAC and PAC systems over the YAC system include lower levels of chimerism, increased stability over high copy vectors (e.g. cosmids) (25), easier to generate libraries, and simpler to manipulate and isolate insert DNA (26).

One primary research focus of our group is to establish and

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implement efficient map-based gene cloning systems for crop plants for the isolation of agriculturally important genes. Map-based cloning is defined as the isolation of a gene based solely on its position on a genetic map and often includes four basic elements: 1) target gene mapping; 2) physical mapping; 3) chromosome walking and 4) gene identification (27,28). Such a strategy has been used successfully for the isolation of a number of important genes from mammals to plants (18–29).

We therefore wanted to test the feasibility of using the BAC cloning system to construct large insert DNA libraries for physical mapping and chromosome walking in crop plants using *Sorghum bicolor* as our model species. Sorghum is the world's fifth most widely produced cereal grain (30). Since it is highly tolerant to drought, sorghum is an essential source of food, feed and fiber in many arid and semi-arid regions including Africa, China, India and the United States. *S.bicolor* molecular genetics has progressed rapidly in the past 5 years and has led to the construction of at least seven RFLP maps (31–37) and the establishment of a transformation system (38). One important finding from the mapping studies has revealed that sorghum and maize are very closely related and that large stretches of genomic DNA are co-linear (31–33). Because sorghum is closely related to maize and maize to other cereals (39), genes isolated from sorghum may be directly transferred into these crops and thus would provide a new and untapped source of genetic variation for crop improvement. Additionally, because of the documented conservation of gene order across many regions of the cereal genomes it may be possible to utilize a *S.bicolor* BAC library as an intermediate species in chromosome walking.

In this report, we describe a detailed account of the construction and characterization of a *S.bicolor* BAC library including; partial digestion of *S.bicolor* megabase-size DNA; library construction, indexing and screening; insert size; chloroplast DNA content; fluorescent *in situ* hybridization; insert stability; and chromosome walking.

MATERIALS AND METHODS

BAC vector preparation

pBeloBACII (7.4 kb) in *E.coli* strain DH10B (F^- *mcr* A Δ (*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 (*ara,leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G) (40) was provided by Drs H.Shizuya and M.Simon, Department of Biology, California Institute of Technology, Pasadena, CA. pBeloBAC II was streaked out onto an LB plate containing 30 μg/ml chloramphenicol (CM), X-GAL (240 μl of 20 mg/ml) and IPTG (24 μl of 200 mg/ml) and grown at 37°C overnight. A single blue colony was used to inoculate 4 l of LB media, pre-warmed at 30°C, containing 30 μg/ml chloramphenicol. The inoculum was grown for 20 h at 30°C to an OD_{600nm} between 1.0 and 1.5, and was then harvested by centrifugation at 6000 *g* for 15 min. Plasmid DNA was isolated from the cell pellet by alkaline lysis using the QIAGEN plasmid Maxi Kit (QIAGEN Cat# 12162, USA) according to manufacturers' specifications. Five QIAGEN tip 500s were used for a 4 l preparation. After the final ethanol precipitation and resuspension in T.E., the plasmid DNA was further purified by cesium chloride density gradient centrifugation in the presence of ethidium bromide in a Beckman (USA) SW50.1 swinging bucket rotor at 35 000 r.p.m. for 72 h. After centrifugation, the plasmid band was removed from the density gradient and the ethidium bromide was removed by several extractions with ddH_2O saturated isoamyl alcohol. The DNA sample was diluted 5-fold with TE and then precipitated with ethanol and centrifuged. The pellet was washed with 70% ethanol, air dried and re-suspended in 500 μl of TE. The final yield was ~50–70 μg of pBeloBAC II DNA from 4 l of media.

pBeloBAC II (20 μg) was digested to completion with *Hin*dIII (Promega, USA) at 37°C for 5 h. Complete digestion was verified on an agarose gel and the DNA was extracted twice with phenol/chloroform followed by a chloroform extraction. The DNA was precipitated with ethanol as above and resuspended in 15 μl of TA buffer (33 mM Tris–acetate, pH 7.8; 66 mM K–acetate, 10 mM Mg–acetate, 0.5 mM DTT, 100 μg/ml BSA) + 5 mM $CaCl_2$. For 1 μg of digested plasmid, 1 unit of HK phosphatase (Epicenter, USA) was added and the DNA was dephosphorylated at 30°C for 1 h. The HK phosphatase was inactivated by heating at 65°C for 30 min followed by the organic extractions and alcohol precipitation as above. The extent of dephosphorlyation was assayed by performing a self ligation test. Ligation of 100 ng of dephosphorylated DNA overnight at 16°C showed no detectable self ligation on an ethidium bromide stained agarose gel. One μg aliquots of the dephophorylated pBeloBAC II plasmid were stored at −80°C until needed.

Plant materials and megabase-size DNA isolation

Sorghum bicolor BTx623 seed was provided by Dr K.Schertz, USDA-ARS, College Station, TX. Plants were grown in flats in a growth chamber with cool white fluorescent lights. Megabase-size *S.bicolor* DNA was prepared in agarose microbeads from protoplasts isolated from leaf tissues of 15–18 day old seedlings (Woo *et al.* in preparation). The protoplasts (6.25×10^8) were embedded in ~10 ml of agarose microbeads and processed as previously described by Wing *et al.* (1993) (41).

BAC library construction

Fifty μl of *S.bicolor* megabase-size DNA (~5 μg) embedded in microbeads was incubated with 100 μl of *Hin*dIII reaction buffer (6 mM Tris–HCl, pH 7.5; 6 mM $MgCl_2$, 50 mM NaCl; 1 mM DTT), 100 μg/ml BSA, 4 mM spermidine on ice for 20 min. Two units of *Hin*dIII was then added and allowed to diffuse into the beads for 20 min on ice. Partial digestion was carried out by incubating the reaction in a 37°C water bath for 5 min. The reaction was stopped by the addition of 1/10 volume of 0.5 M EDTA, pH 8.0. Partially digested *S.bicolor* DNA was separated on a low melting point agarose CHEF gel (42,43) at 6.0 V/cm, with a 90 s pulse, for 20 h, at 11°C running in TAE buffer (50 mM Tris–acetate, pH 7.7; 0.5 mM EDTA pH 8.0). DNA ranging from 300 to 500 kb was cut from the gel, and used for ligation or subjected to a second size selection. A second size selection was carried out in a 1% low melting point agarose gel at 4.0 V/cm, with a 5 s pulse, for 10 h, at 11°C, in TAE buffer. The compressed DNA band was excised from the CHEF gel and stored at 4°C. A gel piece containing size selected DNA was melted at 67°C for 5 min and digested with 1 unit of GELase (Epicentre, USA) per 100 μg of gel by incubating at 40°C for 1 h. After digestion, about 25 ng of size selected *S.bicolor* DNA was ligated to dephosphorylated pBeloBAC II in a molar ratio of 1 (*S.bicolor* DNA) to 10 (vector DNA) in a total volume of 100 μl with 4 units of T4 DNA ligase (USB, USA) in 66 mM Tris–HCl, pH 7.6; 6.6 mM $MgCl_2$; 10 mM DTT; 66 μM ATP at 16°C overnight. After ligation, the ligation mixture was

transferred to a Millipore filter unit (30 000 NMWL) (Cat # UFC3TTK00) which was placed in a 1.5 ml microfuge tube containing 1 ml of TE at 4°C to dialyze out the ligation buffer. The TE was changed 3 times over a 24 h period. One μl of dialyzed ligation material was used to transform 20 μl of *E.coli* ElectroMAX DH10B cells (BRL, USA) by electroporation using the BRL Cell-Porator system at the following settings: voltage: 400; capacitance 330 μF; impedance Low ohms; charge rate: fast; Voltage Booster resistance: 4000 ohms. Transformed cells were resuspended in 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7.0), and incubated at 37°C with shaking at 225 r.p.m. for 1 h and then plated on LB plates containing CM (12.5 μg/ml), X-GAL, and IPTG.

White recombinant BAC clones were picked with a toothpick to a second LB plus CM (12.5 μg/ml), X-GAL, IPTG plate to verify the color selection. White colonies from the second plate were then transferred to either a 96 or 384 well micro titer dish containing 200 or 70 μl of LB freezing buffer (36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 4.4% v/v glycerol, LB) respectively. The microtiter dish was sealed with parafilm and incubated at 37°C for 24 h at which time the wells turned cloudy indicating growth of the cells. The microtiter dish was then stored at −80°C for long term storage. The library was replicated in triplicate and stored in three different −80°C freezers.

BAC library screening

Six Hybond N+ filters (7.5 cm×11 cm) (Amersham, USA) were placed on a Nunc bio-assay dish (24.5 cm×24.5 cm×0.20 cm) (NUNC # 140835, Denmark) containing LB agar, and 12.5 μg/ml CM. Each filter was inoculated with 96 BAC clones using a 96 well replica plating device (Sigma # R 2508, USA), or 384 BAC clones using a 384 well replica plating device (Genetix, UK). When the 96 well replica plating device was used, the inoculation was repeated an additional 3 times with a different microtiter dish each time so that a total of 384 BAC clones were inoculated onto a single filter. After inoculation the plates are incubated at 37°C for 12−18 h until the colonies were about 2−3 mm in diameter. Each filter was processed by placing the filter, colony side up, on Whatman 3 mm filters saturated with the following solutions and for the specified time: 1) 10% SDS, 4 min; 2) 0.5 N NaOH, 1.5 M NaCl, 5 min; 3) 0.5 M Tris−HCl (pH 7.5), 1.5 M NaCl, 1 mM EDTA (pH 8.0), 5 min; 4) 0.5 M Tris−HCl (pH 7.5), 1.5 M NaCl, 1 mM EDTA (pH 8.0), 5 min; 5) 2×SSC, 0.1% SDS, 5 min; 6) 2×SSC, 5 min; and 7) 0.4 N NaOH, 20 min. Finally the filters were washed extensively with 2 times, 1000 ml washes of 5×SSC, 0.1% SDS, for 20 min each wash with shaking. Finally, once the cell debris were removed, the filters were washed with two, 1000 ml washes of 2×SSC for 5 min, each wash.

The library of 13 440 clones was plated and blotted onto 35 filters. The filters were pre-hybridized in a single box with 200 ml of hybridization buffer [1% BSA—fraction V, 1 mM EDTA (pH 8.0), 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS] with 10 μg/ml of sheared and denatured salmon sperm DNA, at 65°C for 4 h (44). The solution was exchanged with fresh hybridization buffer as above and pre-hybridization was carried out for an additional 8 h. One to 4 denatured random hexamer ^{32}P labeled probes (45) were added and hybridization was carried out for 18−36 h at 65°C. After hybridization the filters were washed 3 times for 30 min each wash with increasing stringency washes of: 2×SSC, 0.1% SDS; 1×SSC, 0.1% SDS; and 0.5×SSC, 0.1% SDS. After the last wash, the filters were blotted dry on paper towels, wrapped in plastic wrap, and autoradiographed using Kodak X-OMAT AR film with a single intensifying screen at −80°C for 48−72 h.

DNA probes

Plasmid DNA probes were obtained from: pELII (*S.bicolor* pollen Adh) and pcSh2 (*Z.maize* Sh2) J.Bennetzen; pMSB8 (*S.bicolor* pollen specific cDNA) E.Pè; pSB027, pSB028, pSB033, pSB158 (*S.bicolor* RFLP) A.Paterson; NPI563, NPI578 (*Z.maize* RFLP) Native Plants Incorporate c/o S.Poethig; pBHP20 (chloroplast gene—ndhA), pBPH 134—(chloroplast gene—rbcL), pBHE319 (chloroplast gene—psbA)—J. Mullet.

Recombinant BAC DNA isolation

BACs containing *S.bicolor* DNA were isolated from 5 ml overnight cultures, LB + 12.5 μg/ml chloramphenicol, by alkaline lysis essentially as described by Silhavy *et al.* (1984) (46) with the following modifications. The final DNA pellet was resuspended in 40 μl of T.E. and typically 10 μl was used for restriction digestion analysis.

Clone stability

Four sorghum BAC clones (88A1, 89H9, 63A6 and 83G10) were picked from the library stored at −80°C, and streaked out onto LB plates containing chloramphenicol (12.5 μg/ml), X-gal, IPTG. Single white colonies were picked from each plate and grown in 50 ml LB containing chloramphenicol (12.5 μg/ml) at 37°C overnight. These primary cultures were considered to be the '0' generation. The overnight cultures were diluted 10^6-fold in 500 ml LB with CM (12.5 μg/ml), and grown overnight at 37°C. Serial cultures were made in this way for an additional 4 days, and each passage was considered to represent about 20 generations (25). The DNA from primary cultures (generation 0) and cultures of day 5 (approximately 100 generations) were analyzed by restriction digestions followed by pulsed-field gel electrophoresis.

Fluorescence *in situ* hybridization

Root tips were pre-treated by immersing in ice water for 24 h and fixed in a 3:1 solution of ethanol:acetic acid for 2−3 days. The root tips were stained in 1% acetocarmine solution for 20 min and then squashed in 45% acetic acid. BAC DNA was labeled with biotin-11-dUTP by standard nick-translation reactions. The *in situ* hybridization technique was slightly modified from Rayburn and Gill (1985) (47). About 10 ng of labeled BAC DNA was used for each slide in a hybridization mixture with 50% formamide, 10% dextran sulfate, 2×SSC, 10 μg salmon sperm DNA and excess sorghum Cot-1 DNA. After overnight incubation at 37°C, the coverslips were removed and the slides were washed at: room temperature in 2×SSC for 5 min; 45°C in 2×SSC for 10 min; room temperature in 2×SSC for 5 min and room temperature in 1×PBS (phosphate-buffered saline) for 5 min. The biotinylated probes were detected with avidin-FITC (fluorescein isothiocyanate) (Vector Laboratory, USA). Chromosomes were counterstained with propidium iodide (PI). Images were taken with a Zeiss epifluorescence microscope equipped with a cooled CCD camera (Photometrics PM512), which was controlled by an Apple Macintosh computer. Gray

scale images were captured separately with filters for FITC and PI. The gray scale images were pseudo-colored and merged using computer software. It is worth emphasizing that although a CCD imaging system was used, the signals of the probes were clearly visible by eye through the microscope.

BAC end isolation

Plasmid rescue. To isolate the right ends of insert DNAs, BAC DNA was isolated and digested separately with *Sac*I, *Bam*HI, *Sph*I and *Not*I for 4 h at 37°C. The digested DNAs were loaded side by side on a 1% agarose gel with 0.5×TBE and electrophoresed at 40 Volts for 12−15 h. The gel was Southern blotted onto Hybond N+ and hybridized with ^{32}P labeled pBeloBACII DNA at 65°C. After 12−15 h the blot was washed 3 times for 30 min each wash with increasing stringency washes of: 2×SSC, 0.1% SDS; 1×SSC, 0.1% SDS; and 0.5×SSC, 0.1 % SDS at 65°C followed by autoradiography. The hybridization patterns were compared relative to the *Not*I control to determine which digestion produced an end fragment slightly larger than the *Not*I digest (between 1−5 kb). A digest that produced an end fragment of between 1−5 kb was then self ligated by ligating 1−10 μl (~5−50 ng DNA) of the digest in a total volume of 100 μl with T4 DNA ligase at 16°C for 4−18 h (48). After ligation, 1−2 μl was electroporated into DH10B and transformants were selected on LB plates with 12.5 μg/ml of chloramphenicol. Plasmid DNA was isolated as above and the right end was verified by digestion with the selected enzyme and *Hin*dIII. The right ends generated from the *Bam*HI and *Sph*I digests were amplified from the BAC vector by PCR reaction with M13 forward sequencing primer (5′ GTAAAACG-ACGGCCAGT 3′) and BAC 4 primer (5′ CACTATAGAATA-CTCAAGC 3′).

Inverse polymerase chain reaction (IPCR). To isolate the left ends of insert DNAs, BAC DNA was digested with *Ava*I, *Bam*HI, *Eco*RI, *Kpn*I, *Sac*I and *Sma*I for 4 h at 37°C. One to 5 μl of the digested DNA (5−25 ng of DNA) was self-ligated in a total volume of 20 μl with T4 DNA ligase at 12°C overnight and the circularized-DNA fragments were used as templates for IPCR (49). One μl of ligation material was used for an IPCR reaction in a total volume of 25 μl containing 10 mM Tris−HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl, 0.1 mg/ml gelatin, 200 μM dNTPs, 0.8 μM primers (BAC1: 5′ CTGCAGGCATGCAAGC 3′, BAC2: 5′ GTCGACTCTAGAGGATC 3′), and 1.25 units of *Taq* DNA polymerase. The reactions were initiated by denaturing at 94°C for 4 min followed by 30 cycles of reactions; 94°C, 1 min 30 s for denaturation; 60°C, 2 min for annealing; and 72°C, 3 min for primer extension. In the last cycle, the primer extension step was performed for an additional 7 min (28). To verify the left ends of insert DNAs, the IPCR products were digested separately with *Hin*dIII and the enzyme used for creating the restriction fragments. The digested DNAs were loaded on a gel side by side with uncut IPCR products.

RESULTS

A *S.bicolor* BAC library

pBeloBACII has three unique cloning sites within the beta-galactosidase gene−*Bam*HI, *Sph*I and *Hin*dIII. Previous experiments showed that *Hin*dIII restricts *S.bicolor* DNA frequently with a complete digest giving a size range below 50 kb. To test the BAC system for library construction, *S.bicolor* DNA was partially digested with *Hin*dIII and the DNA fragments were size selected by pulse-field gel electrophoresis (5,50). Figure 1 shows the optimal partial digestion conditions giving DNA in the size range of 200−500 kb using *Hin*dIII. DNA between 300−500 kb was isolated from this gel and ligated to pBeloBA-CII. The ligation was transformed into *E.coli* which produced approximately 1500 white colonies per 1 μl of ligation mixture. Approximately 5568 clones were produced and stored from this ligation. DNA was isolated from 43 clones and analyzed for insert size. The average insert size was 117 kb with a size range between 25 and 307 kb.

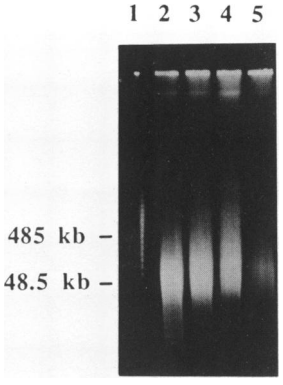

Figure 1. Partially digested *S.bicolor* megabase-size DNA in agarose microbeads. Lane 1: Lambda concatemer (FMC, USA). Lanes 2−5, *S.bicolor* DNA partially digested with *Hin*dIII: 2: 8 units, 3: 4 units, 4: 2 units, 5: 0 units. The DNA was separated by PFG electrophoresis on a 1% agarose gel in 0.5×TBE at 11°C using a Bio-Rad CHEF Mapper at 6.0 v/cm for 20 h with a 90 s pulse time.

In an attempt to increase the average insert size, the remaining DNA from the first size selection, still in the agarose block, was placed in a low melting point agarose gel, size selected at 4.0 V/cm, with a 5 s pulse, for 10 h, at 11°C. After electrophoresis, the compressed DNA band was excised from the CHEF gel, ligated and transformed into *E.coli*. Approximately 150 transformants per μl of ligation reaction was obtained which yielded a total of 7872 BAC clones. Figure 2A shows the analysis of 18 BAC clones derived from insert DNA from two size selections. The DNA was 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 the BAC vector. The remaining bands are *S.bicolor* DNA fragments. Four of 18 insets have no internal *Not*I sites while 14/18 have between 1 to 5 *Not*I sites as seen by multiple bands. Eighty-six BAC clones from the second size selection ligation were similarly analyzed and the average insert for the 7872 BAC clones is ~185 kb with a size range between 50 and 340 kb.

Figure 3 shows a bar graph comparing the insert sizes from the first and second size selections. It is clear that the second size selection eliminated a significant amount of relatively small DNA molecules below 100 kb. The bimodal size distribution for the single size selection may reflect that there was a significant amount of trapping of smaller molecules co-migrating with the selected region in the CHEF gel. We investigated this possibility further by resolving a DNA sample from the first size selection on a CHEF gel under conditions that would spread out the

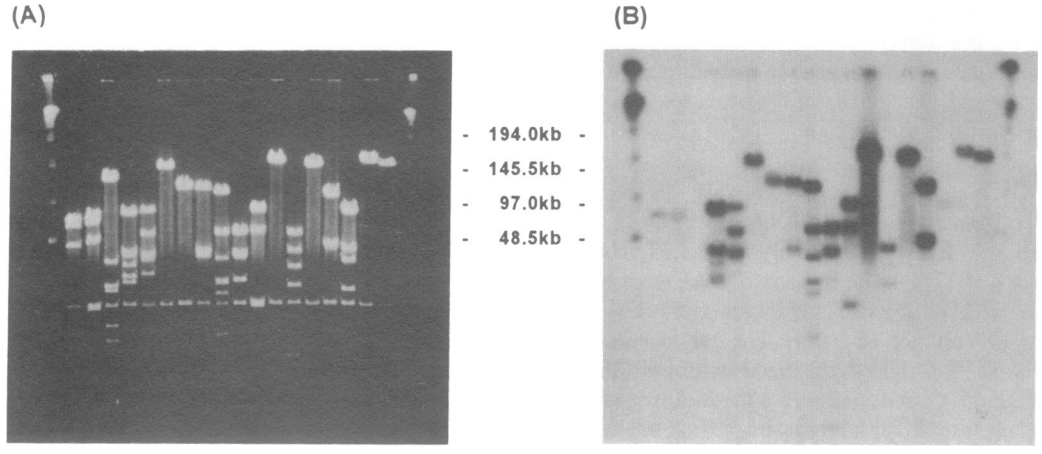

Figure 2. Analysis of *S.bicolor* BAC clones by PFG electrophoresis. **(A)** Ethidium bromide stained CHEF gel. Lanes 1 and 20 Lambda concatemer (FMC, USA). Lanes 2–19, alkaline lysis minipreparations of randomly picked recombinant BAC clones derived from twice size selected insert DNA and digested with *Not*I. PFG electrophoresis conditions, 6.0 v/cm for 17 h, were set with Bio-Rad CHEF Mapper: linear pulse time ramping from 5 to 15 s. **(B)** Southern hybridization of gel in A hybridized with radioactively labeled total *S.bicolor* DNA.

200–300 kb region. The ethidium bromide stained gel showed that the majority of the DNA is in the selected size range however about 10% of the DNA was between 25 and 100 kb. Although on a microgram scale 10% appears low, however on a molar scale 10% comprises ~30% of the ligatable material. Therefore it is extremely important to remove the trapped material by a second size selection before ligation to the BAC vector.

To determine the kind of insert DNA, either genomic—low copy or repetitive, or chloroplast, the gel in Figure 2A was Southern blotted and hybridized with ^{32}P labeled total *S.bicolor* genomic DNA and 3 chloroplast DNA probes. The results in Figure 2B show that 14/18 inserts hybridized with repetitive DNA while 4/18 show very little to no hybridization. These latter clones presumably contain relatively long stretches of DNA, 50–200 kb, uninterrupted with repetitive sequences. Hybridization with total genomic DNA could also be due to the presence of chloroplast insert sequences. Chloroplast DNA content was analyzed by hybridizing the same filter with three chloroplast probes, from *Hordeum vulgare*—ndhA, rbcL and psbA, spaced approximately equally apart such that any BAC insert larger than 60 kb should hybridize with at least one of the three probes. Only two BAC clones (lanes 7 and 17) hybridized with this probe set indicating that these inserts were probably of chloroplast origin. To determine the level of chloroplast sequences in the entire BAC library more precisely, the 3 probes were hybridized to 140 additional BACs. Fourteen percent of the 140 BACs hybridized with the chloroplast probe set.

Clone stability

To evaluate the stability of large plant DNA fragments maintained as BACs in *E.coli*, four large BACs were selected and grown for 100 generations and the restriction digestion and hybridization patterns were compared from the 0 and 100th generation. The four BACs were selected based on size and repetitive DNA content. BACs 88A1 (295 kb), 89H9 (270 kb) and 63A6 (250 kb) were selected because they contain a significant amount of repetitive DNA. When BACs 88A1, 89H4 and 63A6 are digested

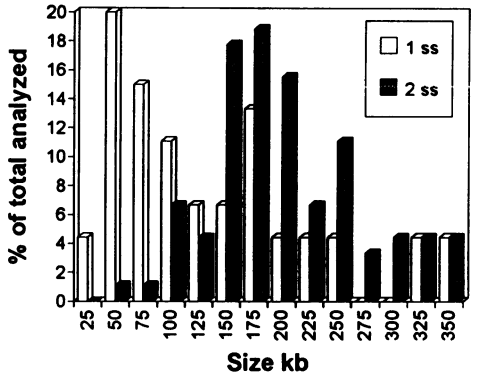

Figure 3. BAC insert size distribution among single (1 ss) and twice (2 ss) size selected *Hin*dIII fragments.

with *Not*I they produce 6, 7 and 5 *Not*I fragments respectively, the majority of which hybridize with radioactively labeled *S.bicolor* DNA. BAC 83G10 (315 kb) when analyzed similarly is relatively free of repetitive DNA sequences. Figure 4A shows the ethidium bromide stained CHEF gel used to resolve the restriction fragments from the 4 BAC clones. Lanes 2,4,6 and 8 contain DNA isolated from the primary cultures (0 generation) and lanes 3, 5, 7 and 9 contain DNA isolated after 100 generations of growth on selective liquid media. Each DNA sample was digested with *Not*I. As shown in Figure 4A, there is no apparent difference in the restriction patterns throughout this experiment indicating at the gross level these four BAC DNAs are stably maintained. These patterns were further verified by identical hybridization patterns for the repetitive elements contained within the *Not*I DNA fragments (Figure 4B). Further analysis of these four BAC clones with two additional restrictions enzymes (data not shown) did not indicate any detectable differences between

any of the BAC DNA samples analyzed at the 0 generation and 100 generation stage. These results imply that various genomic plant DNA sequences are stably maintained as BACs in *E.coli*.

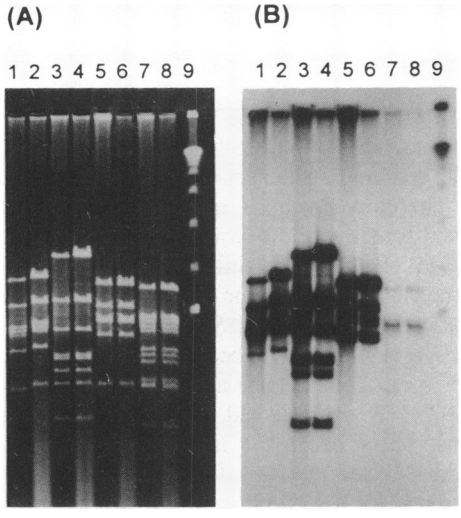

Figure 4. Clone stability. (**A**) Ethidium bromide stained CHEF gel of *NotI* restriction digests of individual BAC clones from before and after 100 generations of growth in liquid medium. Lanes 1–2: BAC 88A1 (295 kb) before (lane 1) and after 100 generations (lane 2); lanes 3–4: BAC 89H9 (270 kb) before (lane 3) and after 100 generations (lane 4); lanes 5–6: BAC 63A6 (250 kb) before (lane 5) and after 100 generations (lane 6); lanes 7–8: BAC 83G10 (315 kb) before (lane 7) and after 100 generations (lane 8); lane 9: Lambda concatemer (FMC, USA). The DNA restriction fragments were analyzed by PFG electrophoresis on a 1% agarose gel in 0.5×TBE at 11°C using a Bio-Rad CHEF Mapper at 6.0 v/cm for 16 h with linear pulse time ramping from 5 to 15 s. (**B**) Southern hybridization of gel in A hybridized with radioactively labeled total *S.bicolor* DNA.

BAC library screening

To estimate the ability to isolate a specific clone or initiate a chromosome walk using the *S.bicolor* BAC library, the library was screened by colony hybridization for the presence of 6 sorghum and 3 maize specific probes. Table 1 summarizes the probes and results of the library screen. At least one BAC clone was isolated for each probe tested except RFLP probe pSB028 (34).

Chimerism estimation by fluorescence *in situ* hybridization

Many large insert DNA libraries contain a significant amount of chimeric clones which can make chromosome walking problematic (51). Therefore determining the level of chimeric clones in the *S.bicolor* BAC library is important. Chimerism can be evaluated by a number of methods including the isolation and genetic mapping of the ends of the cloned fragments or fluorescence *in situ* hybridization (FISH) (23). To estimate the level of chimerism in the *S.bicolor* BAC library eleven of the largest BAC clones detected, 40A5 (220 kb), 52A4 (235 kb), 63A6 (250 kb), 64A3 (330 kb), 83G10 (315 kb), 88A1 (295 kb), 89H9 (270 kb), 124G1 (285 kb), 131N19 (260 kb), 128A3 (250 kb), 131M23 (285 kb), were hybridized *in situ* to both metaphase plate and interphase nuclei. Figure 5A and B show the hybridization patterns of BACs 13IM23 (285 kb) and 83G10 (315 kb) on somatic metaphase chromosomes and an interphase nucleus, respectively. Hybridization signals for BAC 13IM23 are located at the middle of the long arms of a pair of unidentified sorghum chromosomes (Figure 5A). For BAC 83G10, only two spots of intense fluorescence can be detected. The hybridization patterns for both BAC clones are characteristic of clones that are not chimeric. Similar results were obtained with the remaining 9 BAC clones and suggest that the level of chimerism in the *S.bicolor* BAC library is very low.

Table 1. Identification of bacterical artificial chromsome clones corresponding to *S.bicolor* and *Z.maize* RFLP and gene specific probes

Probe	BAC[a] (size in kb)	Source
pSB027 (*S.bicolor* RFLP)	78H7 (40), 128D3 (ND)	Chittenden et al. 1994
pSB028 (*S.bicolor* RFLP)	No BACs	Chittenden et al. 1994
pSB33 (*S.bicolor* RFLP)	41G6 (250)	Chittenden et al. 1994
pSB158 (*S.bicolor* RFLP)	37G7 (85),38H5 (250), 105A17 (190), 133F13 (260), 124E7 (125), 122P8 (245)	Chittenden et al. 1994
pEL11 (*S.bicolor* pollen *Alcohol dehydrogenase*)	51H9 (200), 68A2 (140), 87G9 (60)	J.L. Bennetzen and Chang-Nong Lin, unpublished
pMSB8 (*S.bicolor* pollen cDNA)	40E4 (280), 128D12 190)	M. E. Pè, Universita degli studi di Milano, unpublished
NPI563 (*Z. mays* RFLP)	16H9 (160), 85D6 (135), 132D8 (250)	Pioneer Hi-Bred International
NPI578 (*Z. mays* RFLP)	126P3 (185), 136D5 (130), 146O7 (150)	Pioneer Hi-Bred International
pcSh2-1 (*Z. mays Shrunken*-2)	11C12 (50), 71A1 (80), 86B6 (205)	Bhave et al. 1990

[a]The numbers of the BACs indicate the number and position in the 96 or 384-well master microtiter plate of the *S.bicolor* BAC library.

Chromosome walking

To determine if a specific BAC clone contains a target gene or to continue a chromosome walk it is necessary to isolate the ends of large restriction fragments inserted into the BAC vector. Figure 6 shows a scheme to isolate such ends using plasmid rescue and inverse polymerase chain reaction (IPCR) (15,49). Plasmid rescue can be used to isolate the right ends of the inserts and IPCR is used to isolate the left ends of the inserts.

Plasmid rescue involves restriction digestion of the BAC DNA with an enzyme that cuts several times within the insert and once to several times within the BAC vector followed by re-circularization and transformation into *E.coli*. The resulting plasmid contains only the most extreme end of the insert which does not have the any further internal restriction enzyme sites. The plasmid portion contains DNA sequences essential for replication and antibiotic selection. At present we have used three restriction enzymes for plasmid rescue *Bam*HI, *Sph*I and *Sac*I. To determine which enzyme would be suitable for a particular unknown insert, BAC DNA was first digested with the above enzymes and *Not*I, resolved on an agarose gel, Southern blotted and hybridized with ^{32}P labeled pBeloBACII. The size of the end fragment was then estimated by comparison of the hybridizing band with the hybridizing band in the *Not*I digest. Digests that produce approximate end fragments of between 200 and 5000 bp were diluted 50-fold, self ligated and transformed into *E.coli*. The inserts from the candidate end clones were then amplified by PCR using the M13 forward sequencing and BAC4 primers when *Bam*HI and *Sph*I were used for plasmid rescue. Figure 6B shows the PCR amplification products of six BAC ends isolated by plasmid rescue.

Due to the lack of any convenient restriction enzyme sites on the right side of the *Hin*dIII site, IPCR was used to isolate the left ends of large DNA fragments inserted into pBeloBACII. Two opposing primers were designed immediately adjacent to the left of the *Hin*dIII site of pBeloBACII. This enabled us to use six different restriction enzymes, *Ava*I, *Bam*HI, *Eco*RI, *Kpn*I, *Sac*I and *Sma*I, for the generation of IPCR templates. Figure 6C shows and example of IPCR amplification products produced from the same six BAC clones as above.

DISCUSSION

Large insert DNA libraries are important for map-based gene cloning, the assembly of physical maps and for simple screening for specific genomic sequences in organisms with very large genomes. Therefore methods must be developed to quickly construct and screen such libraries. A primary objective of this work was to evaluate the utility of the bacterial artificial chromosome cloning system for the construction of large insert DNA libraries for crop plants. *S.bicolor* is an ideal crop plant to test the BAC cloning system because of its importance in world agriculture, small genome size and absence of a YAC library. *S.bicolor* BTx623 was selected as the DNA source for BAC library construction because it is the common parent for both interspecific (34) and intraspecific (36) RFLP maps and is the recurrent parent for a recombinant inbred population currently being developed at Texas A & M University (K.Schertz and G.Hart, personal communication).

A *S.bicolor* BAC library

A BAC library of 13 440 clones, from a single and a double size selection, with a combined average insert size of 157 kb was constructed. This insert size is comparable with the available plant YAC libraries, the majority of which have average insert sizes of about 150 kb (9–12). Fourteen percent of the library contains chloroplast sequences, therefore based on a genome size of 750 Mb (52), there is a 91% chance of isolating a specific genomic region from this library if the chloroplast fraction is subtracted

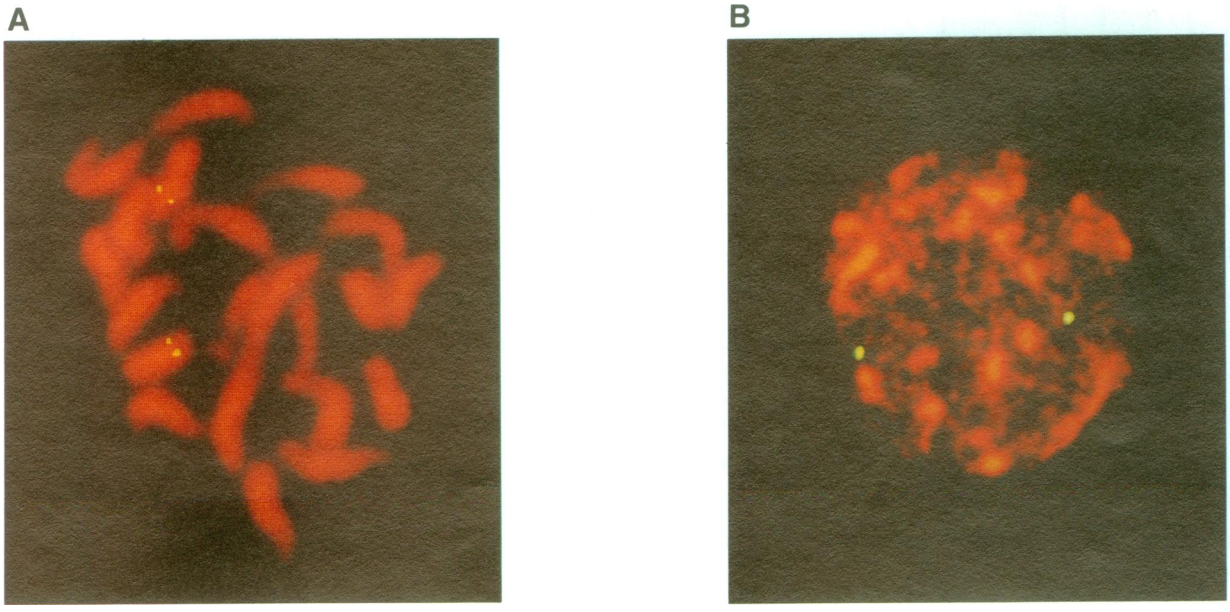

Figure 5. Fluorescence *in situ* hybridization patterns of: (**A**) BAC 13IM23 (285 kb) on somatic metaphase chromosomes; (**B**) BAC 83G10 (315 kb) on an interphase nucleus; Only two hybridization spots were observed in interphase nuclei, and a single pair of metaphase chromosomes had hybridization signals, indicating no chimerism was detected with BACs 83G10 and 13IM23.

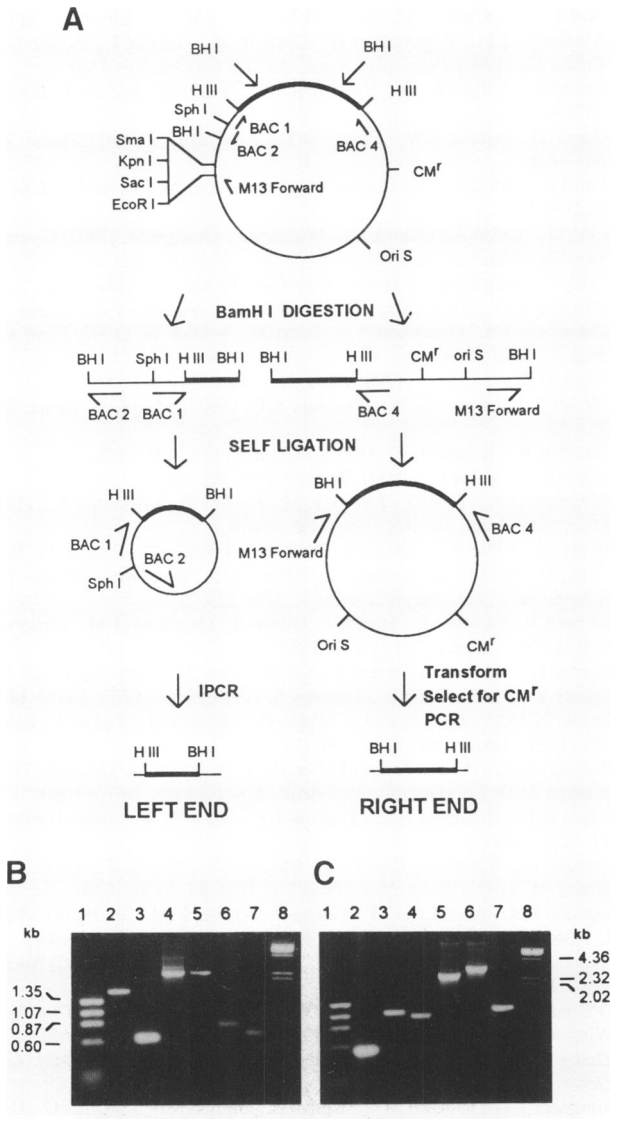

Figure 6. (A) Schematic diagram of the generation of left and right end specific probes from BACs with IPCR (left end) and plasmid rescue (right end) and the restriction enzyme *Bam*HI. BAC 1, 2, 3 and M13 Forward are oligonucleotide primers specific to pBeloBACII. (B, C) Isolation of the ends of *Hin*dIII restriction fragments inserted into pBeloBACII by IPCR (B) and plasmid rescue (C) from six different BAC clones. Lanes 1 and 8 contain molecular size standards: a *Hae*III digest of ϕX174 RF and a *Hin*dIII digest of Lambda DNA, respectively.

out. This number was reflected when the library was screened with six *sorghum* and three *maize* probes (Table 1). Eight of the nine probes hybridized to at least one BAC clone whereas with one RFLP, marker no BAC clone was identified. The clones used to screen the BAC library were selected based on their single copy representation within *S.bicolor* so we were surprised when pSB158 hybridized with six BAC clones. Upon closer inspection of the BAC clones, three hybridized strongly with pSB158 and three hybridized moderately. Further analysis of the pSB158 revealed that this probe hybridizes to two loci on the Sorghum genome—one mapped (34) and one unmapped. Presumably the two classes of BAC clones detected are derived from these two loci.

The sorghum RFLP map must be sufficiently saturated and the inserts in the BAC library sufficiently large for chromosome walking to be feasible. In *sorghum* approximately 500 RFLP markers have been mapped onto 7 linkage maps (31–37). The two most complete maps with 10 linkage groups have a calculated average genetic distance of 1478 cM (34,36). Therefore the average distance from an RFLP marker to a sorghum gene is about 750 kb on either side. Using the current BAC library, if a marker is 1 cM from a target gene, a chromosome walk of at least 3–5 steps would be required. These estimates do not take into account differing recombination frequencies in specific locations and emphasize the need to estimate the relationship between genetic and physical distances before initiating a chromosome walk.

Chromosome walking requires the ability to isolate end fragments which are used as probes to rescreen the library. Additionally, before walking it is important to try to determine the direction of the walk by genetically mapping BAC end. A scheme was developed for the isolation of the ends for chromosome walking by plasmid rescue and IPCR. Unfortunately the majority of ends isolated by this method were repetitive and thus made genetic mapping difficult. The repetitive nature of the ends could be solved by subcloning smaller end fragments and using only single copy sequences for mapping. Additionally it may be possible to map the ends in closely related species such as maize or rice.

An alternative to ordering a group of BACs spanning a few hundred kilobases is to use FISH on interphase nuclei with the whole BAC. This type of ordering has been successfully demonstrated in human using unique sequences and cosmids (53,54). In addition FISH with BACs can be used to determine if the clone is chimeric or not (23). Our results demonstrate that FISH with BAC clones can be used with sorghum chromosomes and is useful for the evaluation of chimerism in both metaphase and interphase preparations.

Evaluation of the BAC cloning system: BACs vs YACs

When evaluating the BAC cloning system a comparison must be made with the proven YAC system.

The primary similarity between the two systems is that they can in principle handle any size of DNA that is cloned into a cloning site. Furthermore both YACs and BACs are maintained as single copy clones in the host cell.

The differences between the two system are broad and can be attributed mainly to the host systems–yeast vs bacteria. *E.coli* divide faster, are easier to isolate DNA from and to transform. All of these factors contribute to the speed and efficiency for the construction and analysis of BAC libraries and individual BAC clones.

For library construction the transformation efficiency of the host cell is critical. For *S.cerevisiae* Burgess and Percivil (1987) (55) reported a system that reproducibly yields competent yeast spheroplasts with transformation efficiencies of $10^7/\mu g$. However in our hands it is difficult to reproducibly make competent yeast cells that are this efficiently transformed. Additionally, the amount of time required to conduct a yeast transformation, which includes growing the cells to the optimal cell density and incubating the cells with the optimal amount of spheroplasting enzyme, is very time consuming. In contrast, the BAC library we constructed used electroporation to introduce the ligated DNA into *E.coli*. 'Homemade' competent cells yield

transformation efficiencies of up to 10^9 transformants/μg and commercially available cells guarantee at least 10^{10} transformants/μg with control plasmids (e.g. pUC19). Under optimal conditions, a 1000-fold difference in transformation efficiencies between yeast and bacteria (10^7 vs 10^{10}) can make a significant difference when the objective is to construct a complete library. Additionally, because of the higher transformation frequencies for *E.coli* the amount of size selected DNA required to make a complete BAC library should be less than that needed for a YAC library. Furthermore, BACs may be more efficiently transformed than YACs because the BAC is circular and probably more stable than a linear YAC.

We should note that we have not recovered recombinant BACs larger than 350 kb. This is in contrast to the YAC system where human and mouse libraries have been constructed with average insert sizes of greater than 600 kb (7). Since *E.coli* can replicate its own genome of about 4 Mb it is unlikely that *E.coli* could not replicate a 1 Mb BAC. One possible reason that we and others have not detected larger BAC clones is that there may be a limit to the size of a molecule that can be delivered by electroporation. It therefore might be possible to create larger insert BAC libraries using conventional $CaCl_2$ transformation techniques (56) or biolistics (57).

Once large insert DNA libraries are constructed the ability to screen for and analyze individual clones efficiently becomes important. Whole YACs have been used as probes for physical mapping by FISH (58) and to isolate genome specific cDNAs (18,19,59). Normally the isolation of YAC probes requires standard megabase DNA isolation in agarose plugs or beads followed by CHEF gel electrophoresis and gel isolation. This process can take 3–5 days to complete. Such isolations do not always guarantee that the YAC can be separated from the 17 additional endogenous yeast chromosomes and YAC DNA yields are often low. In contrast, BAC DNA isolations uses standard plasmid DNA isolation techniques which take advantage of the separation of supercoiled plasmid DNA from bacterial genomic DNA. Figure 2 shows BAC DNA isolated from standard 5 ml minipreparations (46). The DNA preparations take 3 h and the whole process, including CHEF electrophoresis and insert isolation, takes 36–48 h.

In short, the BAC system is more 'user friendly' and will likely lead to more rapid progress in cloning biologically significant genes from crop plants.

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