

Construction of a full bacterial artificial chromosome (BAC) library of *Oryza sativa* genome

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ABSTRACT

We have constructed a full BAC library for the superior early *indica* variety of *Oryza sativa*, Guang Lu Ai 4. The MAX Efficiency DH10B with increased stability of inserts was used as BAC host cells. The potent pBelo BACII with double selection markers was used as cloning vector. The cloning efficiency we have reached was as high as 98%, and the transformation efficiency was raised up to 10^6 transformants / μg of large fragment DNA. The BAC recombinant transformants were picked at random and analyzed for the size of inserts, which turned out to be of 120 kb in length on average. We have obtained more than 20,000 such BAC clones. According to conventional probability equation, they covered the entire rice genome of 420,000 kb in length. The entire length of inserts of the library obtained has the 5- to 6- fold coverage of the genome. To our knowledge, this is the first reported full BAC library for a complex genome.

Key words: bacterial artificial chromosome, *indica*, *Oryza sativa*, library.

INTRODUCTION

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Abbreviations: BAC, bacterial artificial chromosome; SOB medium (without magnesium): Bacto-tryptone 20 g, Bacto-yeast extract 5 g, NaCl 0.584 g, KCl 0.186 g, distilled water to 1 L; WB, 10% ultra pure glycerol, 90% distilled water, v/v; SOC medium: SOB medium with 0.02 M Mg^{2+} and 0.02 M glucose; PFGE, pulsed-field gel electrophoresis.

Representative BAC library of rice genome

The sheer size of the rice genome is the major impediment to the construction of a comprehensive physical map. The need to rapidly clone, map and manipulate large DNA molecules has become acute in conjunction with the rice genome project. Yeast artificial chromosomes (YACs) [1,2] are currently the major technology being employed in the mapping of complex genomes, such as those of human and rice. The major problem with YAC cloning system is its high frequency of *in vivo* recombination. The BAC cloning system [3] offers advantages over the YAC cloning system in three important areas. First, it is possible to prepare BAC libraries that contain very few chimeric clones. Second, it is possible to stably propagate in BAC certain DNA sequences that cause instability and rearrangements in YAC vectors. Third, the isolation and analysis of BAC clones are substantially easier and faster. Adoption of BAC cloning system would thus be expected to achieve significant savings of time and money, associated with the mapping and analysis of complex genomes.

MATERIALS AND METHODS

Materials

14 days old rice, *indica* variety Guang Lu Ai 4 (provided by China National Rice Research Institute, Hang Zhou), growing in green house. Host cell, *E. Coli* strain DH10B (genotype: F⁻ *merA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*dlacZ* Δ M15 Δ *lacX74 deoR racA1 endA1 araD139 Δ (*ara, leu*)7697 *galU galK* λ ⁻ *rpsL nupG*) with high stability of inserts was obtained from GIBCO. Vector pBeloBACII (unpublished) was from Dr. M. Simon. Division of biology, California Institute of Technology, Pasadena, CA, USA, through Dr. Hongbin Zhang of Texas A&M University. pBeloBAC II is a F factor vector, with chloramphenicol resistance gene and *lacZ* gene as selection markers, and unique restriction site, HindIII, among others, as cloning site. β -agarose (DNase free) was from New English Biolabs, Hind III, NotI, lysozyme, T4 DNA ligase, proteinase K, sucrose (molecular biology grade) were from Boehringer Mannheim. Agarose (ultra pure DNA grade, molecular biology certified) from BioRad Laboratories. Pulsed-gel electrophoresis system, chef mapper, including high volt power supply, pulsed-field gel electrophoresis unit, cooling unit, and pulse control unit was purchased from BioRad. Electroporator(with cuvettes), a Cell-Porator *E.coli* system, was from BRL.*

Preparation of large DNA fragments from plant nuclei and digestion of DNA embedded in agarose

Rice leaves were used as materials for the preparation of large DNA fragments by the modification of the method [4-8]. The tissues can be either frozen in liquid nitrogen and stored in a -70°C freezer or kept fresh on ice before use. The reagents used in the preparation were the following. 10 × homogenization buffer (HB) stock: 0.1M Trizma base, 0.8M KCl, 0.1M EDTA, 10 mM spermidine, 10 mM spermine, final pH 9.4-9.5 adjusted with NaOH. The stock is stored at 4°C. 1 × HB: a suitable amount of sucrose is mixed with a suitable volume of 10 × HB stock. The final concentration of sucrose is 0.5 M and HB stock is 1x. The resultant 1 × HB is stored at 4°C. Before use, β -mercaptoethanol is added to 0.15%. Wash buffer (1 × HB plus 0.5% Triton X-100) is prepared by mixing 1 × HB without β -mercaptoethanol with TritonX-100 and stored at 4°C. Before use, β -mercaptoethanol is added to 0.15 %. Lysis buffer: 0.5M EDTA, pH 9.0-9.3, 1% sodium lauryl sarcosine, and 0.1 mg/ml protienase K. The proteinase K powder is added just before use.

Grind about 25 g of the frozen tissue into powder in liquid nitrogen with a mortar and pestle and immediately transfer into an ice-cold 500 ml beaker containing 200 ml ice-cold 1 × HB plus

β -mercaptoethanol and 0.5% Triton X-100. Gently swirl the contents with a magnetic stir bar for 10 min. on ice and filter into an ice-cold 250 ml centrifuge bottle. Pellet the homogenate prepared by the above method by centrifugation with a fixed-angle rotor at 1,800 g at 4°C for 20 min. Discard the supernatant fluid and add approximately 1 ml of ice-cold wash buffer. Gently resuspend the pellet with assistance of a small paint brush soaked in ice-cold wash buffer and finally, add an additional 30 ml of the ice-cold wash buffer. In order to remove the particulate matter remaining in the suspension, filter the resuspended nuclei into a 50 ml centrifuge tube through two layers of gauze by gravity. Centrifuge the contents at 570 g, 4°C for 2 min to remove intact cells and tissue residues. Transfer the supernatant fluid into a fresh centrifuge tube and pellet the nuclei by centrifugation at 1,800 g, 4°C for 15 min. in a swinging bucket centrifuge. Wash the pellet 1-2 additional times by resuspension in wash buffer followed by centrifugation at 1,800 g, 4°C for 15 min. After the final wash, resuspend the pelleted nuclei in a small amount (about 1 ml) of $1 \times$ HB without β -mercaptoethanol, and store on ice. In order to embed the nuclei in agarose plugs, prewarm the nuclei to 45°C in a water bath (about 5 min) before being embedded in agarose. Mix the nuclei with an equal volume of 1% low-melting point agarose in $1 \times$ HB without β -mercaptoethanol using a Pipetman. The agarose should be melted in boiling water and kept at 42°C before use. Aliquot the mixture into ice-cold plug molds on ice with the same Pipetman (100 μ l/plug). When the agarose is completely solidified, transfer the plugs into 5-10 volumes of lysis buffer. Incubate the agarose plugs in the lysis buffer for 24-48 h at 50°C with gentle shaking. Wash the plugs once in 0.5 M EDTA, pH 9.0-9.3 for 1 h at 50°C, once in 0.05 M EDTA, pH 8.0 for 1 h on ice, and store in 0.05 M EDTA, pH 8.0 at 4°C. Before digestion of DNA embedded in agarose, wash the plugs containing high molecular weight DNAs three times in 10-20 volumes of ice-cold TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) plus 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) and three times in 10-20 volumes of ice cold TE on ice, 1 h/each wash. For digestion of agarose plugs, mix the followings in a sterile microcentrifuge tube: Water 31 μ l, agarose plugs of about 50 μ l, $10 \times$ enzyme buffer 10 μ l and 40 mM spermidine 5 μ l. After incubation on ice for 1 h, add 4 μ l (approximately 10 units/ μ g DNA) of restriction enzyme —HindIII was used in this work—for complete digestion and add 4 μ l of each of enzyme dilution series for partial digestion. Incubate on ice for additional 30 min to allow the enzyme to access to the DNA in the agarose and then transfer the reaction mixture to the recommended temperature for enzyme activity. For complete digestion, incubate the reaction mixture for at least 3 h. For partial digestion, incubate the agarose plugs for 1 h. Stop the reaction by adding 1/10 volume of 0.5 M EDTA, pH 8.0. Load the digested high molecular weight DNA in agarose plugs into the well of an agarose gel for PFGE and seal in position with the same agarose as the gel. Set the PFGE program for the best resolution of the DNA ranging from 100-200 kb[9]. After electrophoresis, DNA ranging about 120 to 150 kb was excised from low-melting agarose gel. The agarose slice was dialysed in 3 to 5 volume of TNE (10 mM Tris, 1 mM EDTA, 50 mM NaCl, pH 7.5) at 4°C for at least 1 h and processed to ligation step. For Southern blotting, stain the gel in ethidium bromide for 1 h, destain for a couple of hours, photograph, and nick the DNA in the gel with 60 mJoules of UV light (254 nm). Blot the DNA in the gel onto Hybond-N⁺ membrane (Amersham, USA) with 1.5 M NaCl, 0.4 M NaOH for 40 h. When finished the blotting, take out the membrane, and wash it in $2 \times$ SSC for 10 min with gentle shaking. The membrane can be directly used for Southern blot hybridization or wrapped with Handy Wrap and stored at 4°C.

Ligation and electroporation

After dialysis, the low melting point agarose slices were melted at 68°C for 10-15 min. Then transfer the tube containing the melted agarose into 40°C bath. Add 1/10 vol. of $10 \times$ agarose buffer (100 mM Bis-Tris-HCl, 10 mM EDTA pH6.5) prewarmed at 40°C and 1 μ l agarose (1 μ) per 100 μ l of melted agarose. The mixture was incubated at 40°C for 1-2 h. One to three hundred nanograms of the rice DNA was ligated to Hind III digested pBeloBAC II (molar ratio of 6 to 1 in pBeloBAC II excess) with T4 DNA ligase at 16°C. overnight[10]. DH10B cells suitable for

Representative BAC library of rice genome

high-efficiency transformation by electroporation[11] can be prepared from log-phase cultures by washing the cells twice and resuspending them at a high cell density (about 1×10^6 cells/ml) in 10 % glycerol. The transformation efficiency (transformants/ μg DNA) in our experiment was about 10^6 . The detailed procedures were as follows. Use a fresh colony of cells to inoculate 50 ml of SOC medium. Grow cells with vigorous aeration overnight at 37 °C. Dilute 0.5 ml of cells into 500 ml of SOC medium in a 2.8-L flask. Grow for 2 to 3 h with vigorous aeration at 37°C until $\text{OD}_{500}=0.8$. Harvest cells by centrifuging at 5,000 rpm ($2,600 \times g$) for 10 min. Wash the cell pellet by resuspending in 500 ml of sterilized, ice-cold WB. Centrifuge the cell suspension at 5,000 rpm ($2,600 \times g$) for 15 min and carefully pour off the supernatant as soon as the rotor stops. Wash the cell pellet once again as above. Resuspend the cell pellet in WB to a final density of 200 to 250 OD_{550} units (about 1 to 2 ml). Usually, no additional WB needs to be added to the cell pellet, it can be resuspended in the WB that remains in the centrifuge bottle. Cells can be used immediately or can be frozen in 200 μl aliquots in microcentrifuge tubes using a dry ice-ethanol bath. Store frozen cells at -70 °C. Aliquot 20 μl samples of cells (not frozen) into sterilized microcentrifuge tubes. Add 1 μl of ligated DNA, and mix with the pipette tip. Use a micropipetter to suspend each cell-DNA sample between the electrode bosses of a Disposable Micro-Electroporation Chamber, and perform electroporation. Extra care in the preparation of DNA for high-voltage electroporation into the host cell will improve both transformation efficiency and consistency. For preparing DNA from ligation reactions, to a 20 μl ligation mixture, add 2 μl of 3 M sodium acetate (pH 7.0), and mix. Add 44 μl of 95% ethanol, and mix. Centrifuge for 15 min at room temperature at $12,000 \times g$ in the microcentrifuge. Remove the supernatant, add 60 μl of 70 % ethanol, and centrifuge for 15 min. Remove the supernatant. Dry the pellet, and resuspend the pellet in 20 μl of $0.5 \times \text{TE}$. Transfer the resuspended DNA to a new microfuge tube, and add to cells. With the VOLTAGE RANGE selector switch on the *E. coli*, set Pulser to MEDIUM (about 2.5 kV), the fixed capacitance of 2 μF , and internal parallel resistance of 4 $k\Omega$, the host cell-DNA mixtures yielded consistently high transformation efficiencies of around 10^6 transformants/ μg DNA.

We have also embedded the nuclei in agarose microbeads and digested the DNA in them. The results were satisfactory.

RESULTS

We have obtained more than 2×10^4 BAC clones which constitute our rice genome library. The digestion of the BAC DNA with NotI to completion was performed in the usual way[5], except that the incubation at 37 °C lasted at least for 3 h, and extra incubation at 65 °C for 10 min was made before loading. For the better resolution for DNA fragments ranging from 10 to 350 kb, we chose the CHEF MAPPER of BioRad, and set the working conditions as follows: 1% agarose, $0.5 \times \text{TBE}$, initial 5 seconds, final 15 seconds, angle 120°, 6v/cm, 12°C, 18 h and linear.

Fig A showed the analysis of rice BAC library constructed in this work. 19 BAC clones were picked up at random, and the DNAs prepared from them were digested with NotI to completion. Lambda ladder and lambda / HindIII digest were on the both side of the gel as molecular markers. Lambda ladders were prepared from lambda by joining their cohesive ends at random, which resulted in concatemers. The BAC inserts were shown to contain various number of NotI sites. The common bands in all lanes except the marker lanes were the vector DNA, which were released from the two unique NotI sites located in the polylinker at the either side of the insert. Lane 1 to 19 were the complete NotI digests of the BAC clones. It can be seen from the figure that the average size of the inserts was of 120 kb in length.

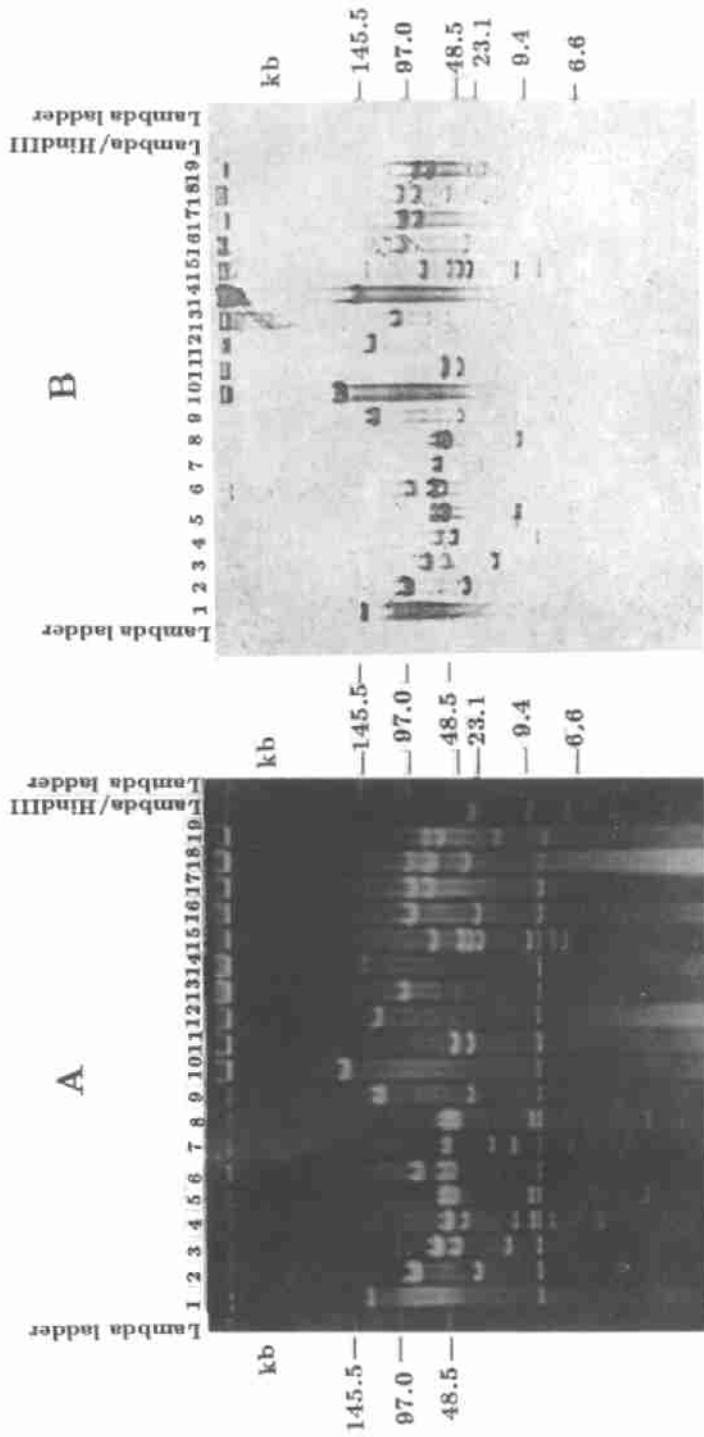


Fig A-B, Analysis of BACs.

A: the DNAs of 19 BACs of Guang Lu Ai 4 were digested with Not I to completion and subjected to PFGE as described in the text.

B: Southern blotting of the gel shown in Fig. A, with total DNA of Guang Lu Ai 4 labeled with dig-dUTP as a probe. The molecular sizes in the Fig A-B are indicated in kb, which were prepared either by annealing of λ DNA at random, or by complete digestion of λ DNA with Hind III.

Representative BAC library of rice genome

Fig B showed the Southern blotting with digoxigenin-dUTP randomly labeled chromosome DNA as probe against the complete digest of the same BAC clones as those shown in Fig A. It can be seen from the figure that all the inserts released from the clones were hybridized with the labeled Guang Lu Ai 4 chromosome DNA from which the BAC library was constructed. No vector DNA and marker DNAs were shown to be hybridized with the chromosome DNA. The result clearly showed that all the inserts of the BAC clones were coming from the rice chromosome, from which the BAC library was made.

DISCUSSION

For construction of a representative library of rice genome, the BAC cloning system was obviously an ideal one. The average insert size of BAC recombinants constructed in this lab was of 120 kb in length. A BAC library containing 1.6×10^4 recombinants with above average size of inserts represents 99% of probability of covering the entire genome. The BAC cloning system is capable of maintaining large fragments of foreign DNA. It has been proved that individual clones of foreign DNAs were maintained with a high degree of structural stability in the host cell, even after 100 generations of serial growth[3]. Because of high cloning efficiency and easy manipulation of the inserted DNA, the BAC cloning system was proved to facilitate construction of the libraries of rice genome with fuller representation. It has been known that chimeric clones were caused by *in vivo* homologous recombination and the wrong ligation *in vitro*. It has been generally believed that about 95 % of the chimeric clones were caused by *in vivo* recombination and the rest caused by the *in vitro* wrong ligation. The use of the host cell with high stability of insert, such as DH10 B, should be expected to have very few chimeric clones, although we did not test the recombinant frequency by observation of a positive signal at two distinct chromosomal locations. In addition, we had taken extreme care in choosing the right PFGE conditions and in cutting out the gel slice, so that no small DNA fragments could contaminate the DNA we want to clone in the BAC system.

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