

TECHNICAL ADVANCE

Construction of a rice bacterial artificial chromosome library and identification of clones linked to the *Xa-21* disease resistance locus

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Summary

A bacterial artificial chromosome (BAC) library consisting of 11 000 clones with an average DNA insert size of 125 kb was constructed from rice nuclear DNA. The BAC clones were stable in *E. coli* after 100 generations of serial growth. Transformation of the BAC clones by electroporation into *E. coli* was highly efficient and increased with decreasing size of the DNA inserts. The library was evaluated for the presence of organellar, repeated, and telomeric sequences. A very low percentage (<0.3%) of the library consisted of chloroplast and mitochondrial clones. Eighteen BACs were identified that hybridized with an *Arabidopsis* telomere repeat. Sixteen BACs hybridized with the AA genome-specific repetitive sequence *pOs48*. Twelve clones were isolated that hybridized with three DNA markers linked to the *Xa-21* disease resistance locus. The results indicate that the BAC system can be used to clone and manipulate large pieces of plant DNA efficiently.

Introduction

Rice (*Oryza sativa* L.) is one of the world's leading food crops, which sustains life in most developing countries. A long tradition of genetic research in rice has led to characterization of many genes which have been utilized to increase rice yield greatly (Khush and Toenniessen, 1991). Recently, two high-density rice molecular genetic maps containing 722 and 1500 markers and covering approximately 1500 cM have been developed (Causse *et al.*, 1994; Shomura *et al.*, 1994, respectively). Many genes affecting important agronomic traits such as disease resistance and drought tolerance have been located on these maps (Champoux *et al.*, 1995; Ronald *et al.*, 1992; Wang *et al.*, 1994). The saturated molecular maps, the small

DNA content (Arumanagathan and Earle, 1991) and large percentage of low copy DNA (Deshpande and Ranjekar, 1980; McCouch *et al.*, 1988) make rice a model monocot for molecular genetic studies and map-based cloning of agronomically important genes.

One of the essentials for the success of map-based cloning and physical analysis of large chromosomal regions is the availability of libraries containing large inserts of genomic DNA. In recent years yeast artificial chromosome (YAC) libraries have been constructed for human (Burke *et al.*, 1987), mouse (Chartier *et al.*, 1992) and plant species, such as *Arabidopsis* (Grill and Somerville, 1991; Ward and Jen, 1990), tomato (Martin *et al.*, 1992), maize (Edwards *et al.*, 1992), barley (Kleine *et al.*, 1993) and japonica rice (Umehara *et al.*, 1995). These libraries have made valuable contributions to the production of physical maps of large regions and the isolation of many important genes (Arondel *et al.*, 1992; Giraudat *et al.*, 1992; Leyser *et al.*, 1993; Martin *et al.*, 1993). However, the large percentage of chimeric (Green *et al.*, 1991; Libert *et al.*, 1993; Umehara *et al.*, 1995) and unstable (Dunford *et al.*, 1993; Neil *et al.*, 1990; Schmidt *et al.*, 1994) YAC clones have hindered the usefulness of these libraries. Difficulties in isolation of YAC insert DNA and low transformation efficiency also affect YAC library construction. An alternative large DNA cloning system based on the bacteriophage P1 has also been developed (Sternberg, 1990). However, the maximum cloning capacity of a P1 library is 100 kb.

Recently, Shizuya *et al.* (1992) described a bacterial artificial chromosome (BAC) system to clone large DNA fragments of the human genome. This system utilizes an F-factor-based vector and is capable of maintaining human genomic DNA fragments of >300 kb. Compared with YAC cloning, DNA can be cloned with high efficiency, manipulated easily and stably maintained in *Escherichia coli* (Shizuya *et al.*, 1992). Application of the BAC cloning system to plant species would greatly facilitate map-based cloning efforts. We report here the construction, characterization and utilization of a BAC library containing 11 000 rice genomic clones with an average insert size of 125 kb. Based on a haploid genome size of 450 Mb (Arumanagathan and Earle, 1991), this library contains three haploid genome equivalents and represents a 95% probability of containing any specific single-copy

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sequence. The completeness of the library was evaluated by probing with rice repetitive DNA, *Arabidopsis* telomere, and monocot organellar sequences. In addition, we have isolated 12 BAC clones that carry DNA fragments linked to the rice *Xa-21* disease resistance locus.

Results

Construction of the library

The library was constructed from high molecular weight (HMW) DNA isolated from nuclei in which more than 95% of chloroplasts and mitochondria were removed during the isolation of nuclei. Initially, we attempted to use HMW DNA embedded in agarose beads (Wing *et al.*, 1993) for library construction. This method was unsuccessful, presumably due to low DNA concentration in the agarose beads. Because of this difficulty, we embedded rice nuclei in agarose plugs (Ganal *et al.*, 1989; Schwartz and Cantor, 1984) and obtained a higher DNA concentration (approximately 5 µg DNA/80 µl plug). DNA was released from the agarose matrix by melting the plugs before partial digestion with *Hind*III to ensure exposure of all the DNA to the enzyme. At least five times more enzyme was required to achieve a similar degree of partial digestion using unmelted plugs. The optimal amount *Hind*III was determined empirically. Three to seven units produced the maximum amount of DNA in the 250–350 kb range and were used in the test ligation and transformation experiments. Five units of *Hind*III per plug yielded the highest percentage of white colonies after ligation to the vector and transformation. Therefore, this enzyme concentration was used for the library construction.

The rice BAC library consists of 11 000 clones. The library was constructed using two different approaches. The first 7269 BAC clones were made with one size selection using a compression zone method (Ramsay and Wicking, 1991). The second half of the library (3731 clones) was made using double size-selection of partially digested DNA. Double size-selection failed to increase the average DNA insert size. This result may be due to small DNA molecules still present in the size-selected DNA solution (only 250–350 kb DNA isolated). Subsequent experiments demonstrated that double size-selection of DNA between 350–500 kb for ligation yielded larger average insert size in BAC clones (data not shown). Out of 54 random BAC clones chosen from the library, 50 clones contained rice DNA (93.0%). Some of the clones (7%) contained no inserts. The size distribution of these clones is shown in Figure 1. The DNA insert sizes ranged between 30 and 250 kb with an average of 125 kb. Seven of these clones were digested with the restriction enzyme *Not*I and the DNA fragments were separated using pulse-field gel electrophoresis (PFGE) (Figure 2a). All clones had at least two *Not*I restriction

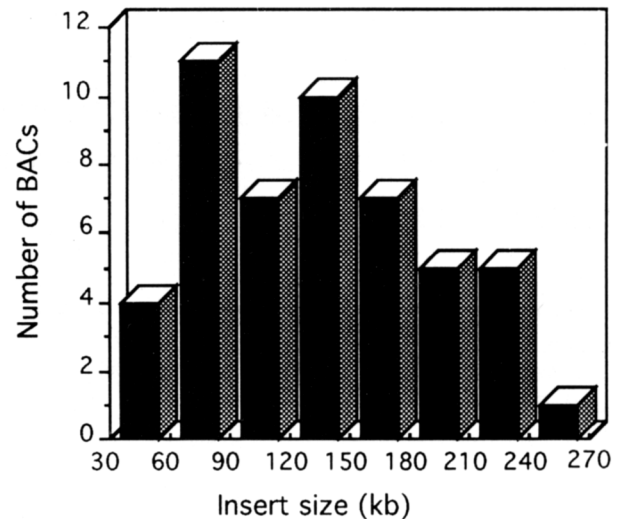


Figure 1. Size distribution of clones in the rice BAC library. Fifty random rice BAC clones were isolated and sized using PFGE.

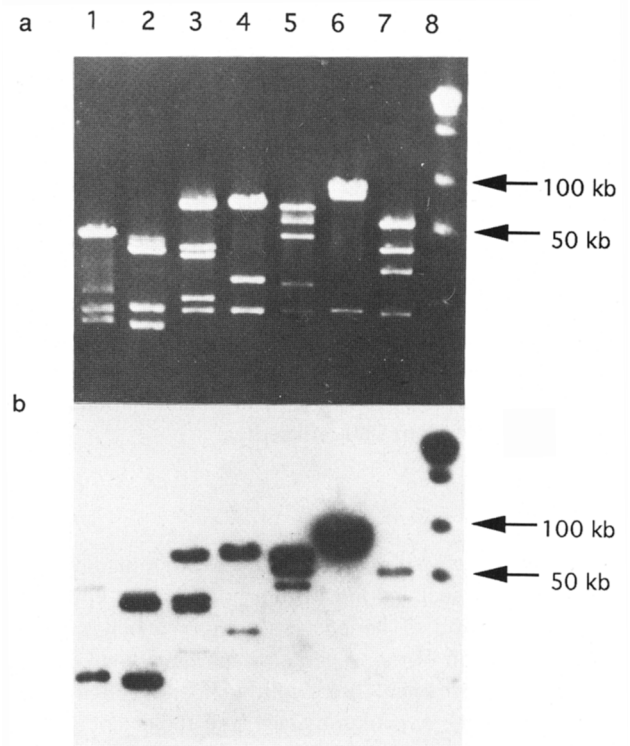


Figure 2. Rice BAC clones hybridize with rice DNA. (a) Ethidium stained agarose gel showing seven random rice BAC clones digested with *Not*I and separated by PFGE (lanes 1–7). Arrows indicate the sizes of DNA fragments deduced from lambda concatamer size markers (lane 8) and the 7 kb pBelo BACII vector. The size of the BACs range from 40 to 210 kb. (b) An autoradiogram showing Southern blot analysis. The gel in (a) was transferred to nylon and hybridized with total rice genomic DNA.

enzyme sites. The average size of the *Not*I DNA fragments is about 50 kb. The reason why *Not*I cuts fairly frequently in the rice genome is probably due to the high GC content

(42%) and low c-methylation (18.5%) of rice DNA (Wu *et al.*, 1992). All seven clones hybridized with total rice genomic DNA (Figure 2b). The strong hybridization signal observed in lane 6 is probably due to hybridization with repeated sequences.

Characterization of the library

To investigate the occurrence of rice repetitive sequences in the library, four genome-specific repetitive sequences (*pOs48*, *pOa4*, *pOo2* and *pOb1*; Zhao *et al.*, 1989) were used to screen a portion of the library containing 1152 BAC clones. No clones hybridized with *pOa4*, *pOo2* and *pOb1* which are EE, CC and FF genome-specific, respectively (the library was made from an AA genome cultivar). Sixteen clones (1.4%) hybridized with the AA specific sequence, *pOs48*. The frequency with which we have recovered the *pOs48* sequence in the BAC library is similar to that reported by Zhao *et al.* (1989).

Since HMW DNA was isolated from nuclei, the frequency of clones containing organellar DNA should be much lower than in libraries made from DNA extracted from protoplasts. In order to determine the percentage of BACs containing organellar DNA sequences, we probed 1536 BACs with genes from the maize and rice chloroplast and maize mitochondria genomes. Four BACs (0.3%) hybridized with the maize chloroplast ribulose biphosphate carboxylase (RBCL) gene. To confirm these results, we used two rice chloroplast clones, i.e. P1 (19.2 kb) and P4 (14.4 kb), to screen the library. Both are *Pst*I clones and contain several rice chloroplast genes (Hirai and Sugiura, 1988). Only two out of 1536 clones hybridized with P1 and P4 clones. Two BACs hybridized with the maize mitochondrial cytochrome oxidase (*COI*) gene. All hybridizing clones were confirmed by Southern blot analysis (data not shown). These results indicate that the rice BAC library has a very low percentage (<0.3%) of clones carrying chloroplast and mitochondrial sequences.

To isolate telomeric clones, a synthesized sequence corresponding to an *Arabidopsis* telomeric repeat (TTAGGG)₁₀ (Richards and Ausubel, 1988) was used to screen 3072 BAC clones. Eighteen clones (0.6%) hybridizing with this sequence were identified and confirmed by Southern blot analysis. Based on the *Hind*III restriction digestion and hybridization patterns, at least four classes of telomeric clones were isolated (data not shown).

Stability of the BAC clones in *E. coli*

In order to examine the stability of rice BAC clones in *E. coli*, an experiment was conducted using two large rice BACs (140 and 210 kb). The DNA from these BACs was extracted after 1 and 5 days of culture and digested with *Xho*I, *Not*I and *Sfi*I. The restriction patterns after 1 and 5

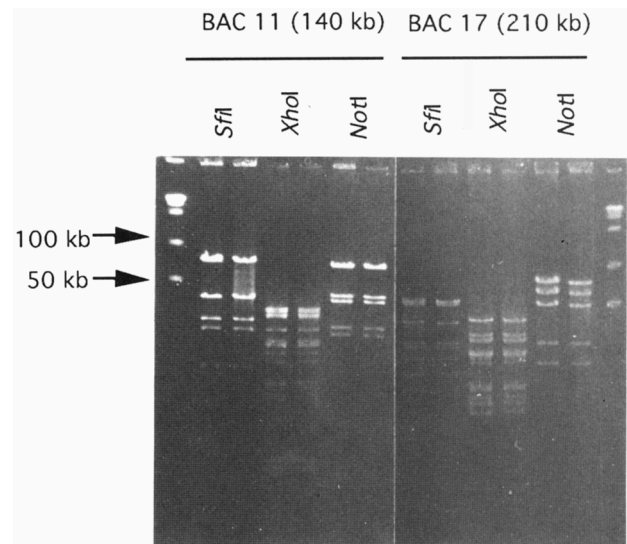


Figure 3. Comparison of the restriction enzyme patterns of two BAC clones (140 and 210 kb) before and after 100 generations of culturing. The first and second lane of each pair represent DNA extracted after 1 day and 5 days of culturing, respectively.

days of culture were compared using PFGE. No visible difference were detected between the two samples (Figure 3). The above results were confirmed using *Hind*III, in which more than 20 fragments were generated for each clone (data not shown). In addition, we have not detected any rearrangement in the approximately 20 clones we have analyzed in detail. These results indicate that plant DNA can be stably maintained as BACs in *E. coli* after 100 generations (5 days) of culture. Similar results were obtained with BAC clones containing human DNA (Shizuya *et al.*, 1992).

Effect of DNA insert size on BAC transformation efficiency

Vector DNA (7.0 kb) and seven BAC clones ranging from 40 to 220 kb were used to determine the relationship between *E. coli* transformation efficiency and DNA insert size. The number of transformants per femtomole of DNA versus plasmid size (insert plus vector) was plotted on a semilog scale (Figure 4). We found that the transformation efficiency decreased when the DNA insert size was increased. For example, a BAC clone of 107 kb (9.6×10^5 transformants fmol^{-1}) had a 13.5 times higher transformation efficiency than a clone of 220 kb (7.1×10^4 transformants fmol^{-1}).

Screening the library with DNA markers linked to the disease resistance locus, Xa-21

One of our purposes in constructing the BAC library is to use it for constructing a contiguous set of clones (contig

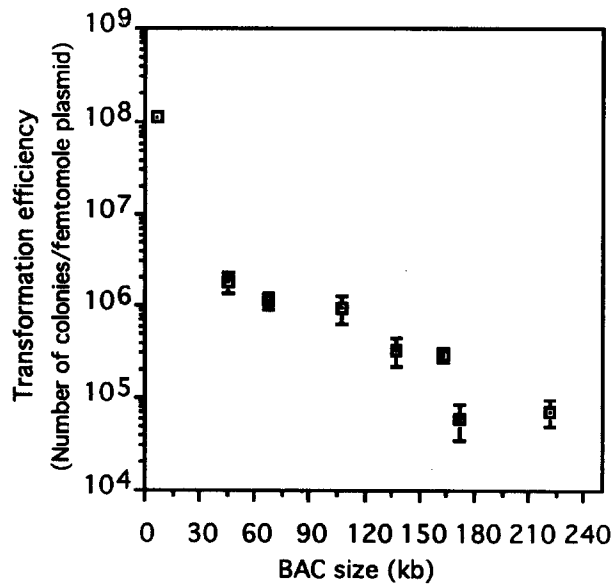


Figure 4. Effect of DNA insert size on BAC transformation efficiency. Seven BAC clones of different sizes (40–220 kb) and the BAC vector (7 kb) were used in the experiment. The number of transformants per femtomole DNA versus plasmid size (insert plus vector) were plotted on a semilog scale.

spanning the *Xa-21* locus. We used two *Xa-21* linked DNA markers, i.e. *RG103* (1 kb) and *pTA818* (1.2 kb, equivalent to *RAPD818* in Ronald *et al.*, 1992), to screen the BAC library. *RG103* is found in eight copies in the *Xa-21*-containing line and hybridizes with eight genomic *HindIII* DNA fragments in this line. All of these fragments are genetically and physically linked to the *Xa-21* disease resistance locus (Ronald *et al.*, 1992; unpublished results). *pTA818* hybridizes with two DNA fragments and at least one of these fragments is linked to the *Xa-21* locus (Ronald *et al.*, 1992). To date, we have probed 7296 BAC clones with *pTA818* (two copies) and *RG103* (eight copies). Seven and five BAC clones hybridizing with *RG103* and *pTA818*, respectively, were identified. BAC DNA was isolated from these clones and digested with *HindIII*. Southern analysis showed that the seven *RG103* hybridizing BAC clones carried four different copies of the *RG103* genomic *HindIII* fragments (Figure 5). Four BAC clones were isolated that carried one copy of the *pTA818* *HindIII* fragment and one BAC clone was identified that contained the other copy (data not shown). One of the *pTA818*-containing BACs also hybridized with the marker *pTA248* (equivalent to *RAPD248* in Ronald *et al.*, 1992), confirming that these two cloned RAPD markers are within 60 kb of each other (Ronald *et al.*, 1992).

The identification of 12 BAC clones hybridizing with two cloned DNA sequences (corresponding to 10 DNA fragments in the rice genome) is slightly lower than the 20 clones expected based on screening 2× genome equivalents (7296 clones, 450 000 kb genome, 125 kb average

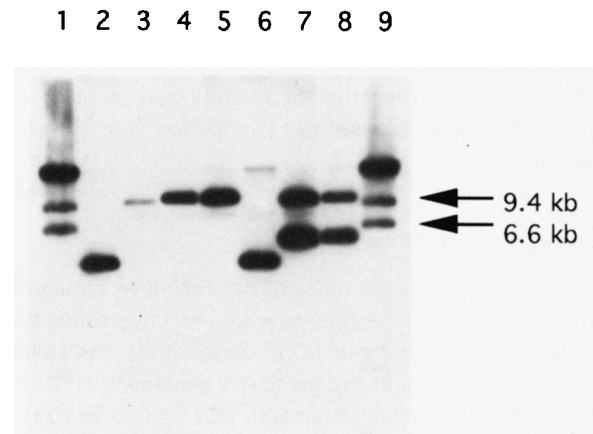


Figure 5. Autoradiogram showing BAC clones hybridized with the *Xa-21*-linked eight-copy marker *RG103*. Plasmid DNA, isolated from bacterial colonies hybridizing with *RG103*, was digested with *HindIII* and the DNA fragments separated by PFGE. The gel was blotted and hybridized with *RG103* to confirm the identity of the clones. The probe hybridized with a 4.3 kb DNA fragment (lanes 2 and 6), a 9.5 kb fragment (lane 3), a 9.6 kb fragment (lanes 4, 5, 7 and 8) and a 6.2 kb fragment (lanes 7 and 8). The sizes of the DNA fragments are deduced from lambda DNA digested with *HindIII* (lane 9).

insert size). Specifically, the *pTA818* sequences and four (out of eight) of the *RG103* hybridizing sequences are overrepresented in this portion of the library. In contrast, the other four *RG103* hybridizing sequences are underrepresented. The DNA insert sizes of these clones ranged from 40 to 140 kb (data not shown). We are currently screening the remainder of the library with these two markers and constructing a contig spanning the *Xa-21* locus.

Discussion

Low frequency of chloroplast and mitochondria clones in the library

The currently available YAC libraries in tomato, barley, maize, *Arabidopsis* and rice were made from HMW DNA isolated from leaf protoplasts which include the cytoplasmic genomes. Most of the libraries have a high percentage (10–26%) of chloroplast clones (Edwards *et al.*, 1992; Martin *et al.*, 1992; Schmidt *et al.*, 1994). Martin *et al.* (1992) found that one YAC clone of tomato hybridized to both a chloroplast gene and a nuclear repeated sequence and may be the result of a co-ligation event. The EG library of *Arabidopsis* (Grill and Somerville, 1991) contains a large portion of chloroplast clones (26%), in which up to 23% of the chloroplast clones may be chimeric with other classes of repeated DNA sequences (Schmidt *et al.*, 1994). The occurrence of chimeric YAC clones greatly reduces the efficiency of chromosome walking experiments and hampers the construction of physical maps. HMW DNA used

to construct the rice BAC library was isolated from purified rice nuclei. Most of the chloroplasts and mitochondria were removed by low speed centrifugation (<1000 *g*). The low frequency of chloroplast or mitochondrial clones found in our rice BAC library (<0.3%) will reduce the possibility of organellar/nuclear DNA co-ligation.

Chimeric clones can also result from the ligation of more than one DNA insert fragment to the vector. To evaluate the frequency of co-cloning events among the BAC clones, 10 random rice BAC clones were mapped in the rice genome by using fluorescence *in situ* hybridization (FISH) techniques. All clones mapped to a single site in the rice genome (Jiang, Gill, Wang, Ronald and Ward, unpublished results).

Telomeric BAC clones

Telomeres are the terminal sequences of linear chromosomes and play a role both in the stability and replication of chromosome ends (Wu and Tanksley, 1993). Telomeric sequences of *Arabidopsis* have been cloned and shown to hybridize with diverse plant species (Richards and Ausubel, 1988). In *Arabidopsis*, there are up to 350 7-bp telomeric blocks present at each telomere (Richards and Ausubel, 1988). Wu and Tanksley (1993) mapped the *Arabidopsis* sequences to the telomeres of three rice chromosomes. We have isolated 18 rice BAC clones that hybridize with a synthesized telomeric sequence. The different restriction enzyme patterns observed for these clones suggest that they may contain telomeres of varying sizes from different rice chromosomes. Alternatively, since it is not known if there are *Hind*III sites located between telomeric repeats at the end of rice chromosomes, some or all of these clones may contain non-telomeric DNA. For example, a satellite band of the *Arabidopsis* telomeric sequences mapped to an internal region of rice chromosome 10 (Wu and Tanksley, 1993). FISH mapping to determine the chromosomal location of the rice 'telomeric' BAC clones is in progress. If the BAC clones do contain telomeres, single-copy sequences from these clones could be used as markers for mapping the ends of the molecular linkage map. In addition, further analysis of these clones may be useful for understanding the structure and organization of the telomere region of plant species.

BAC transformation efficiency

Electroporation is now commonly used as a highly efficient procedure for transforming *E. coli*. Shizuya *et al.* (1992) successfully used electroporation for transforming large human BAC clones. We investigated the relationship between BAC insert size and electroporation efficiency in *E. coli* and found that BACs of 100–200 kb can be transformed efficiently (about 10^6 – 10^5 transformants

fmol^{-1} , corresponding to 10^8 – 10^7 transformants μg^{-1}). The BAC vector (7.0 kb) can be transformed at 10^8 transformants fmol^{-1} , corresponding to 10^9 transformants μg^{-1} . The high transformation efficiency of BAC clones compared with YAC clones greatly facilitates construction of large DNA insert libraries. Martin *et al.* (1992) found that transformation efficiency of the 6.0 kb plasmid YCp50 into yeast was only 0.5 – 1.0×10^6 transformants μg^{-1} . The 1000-fold difference of transformation efficiency between bacteria and yeast (10^9 versus 10^6 transformants μg^{-1}) makes the BAC cloning procedure less time consuming as well as less expensive for construction of complete libraries. For some ligations, we obtained about 200–300 BAC clones with average DNA inserts larger than 100 kb from transforming 1 μl of ligation mixture (data not shown). Thus, a BAC library of 15 000 clones with 100 kb insert size could be constructed from one ligation (100 μl) and 50–80 transformations.

Similar to YAC cloning (Edwards *et al.*, 1992), preferential transformation of small BAC clones lowers the frequency of transformation of BAC clones containing larger inserts. We have demonstrated that the transformation efficiency per mole decreases with the increasing size of DNA inserts. A BAC clone of 220 kb was transformed 13.5 times less efficiently than a 107 kb BAC clone. Little is known about the mechanisms governing the efficient transfer of large DNA molecules into the cell during electroporation (Leonardo and Sedivy, 1990). Although smaller clones are recovered at a higher frequency, 300 kb BAC clones are readily obtainable using this system (Shizuya *et al.*, 1992; Wing, Texas A&M, personal communication).

Usefulness of BAC clones for chromosome walking in rice and other cereal crops

Several genes have been isolated from *Arabidopsis* and tomato, using a map-based cloning strategy (Arondel *et al.*, 1992; Giraudat *et al.*, 1992; Martin *et al.*, 1993). Until recently, such techniques have been limited in rice due to the lack of large insert DNA libraries. With the availability of the rice YAC (Umehara *et al.*, 1995) and BAC libraries, rapid progress is expected for isolation of agronomically important genes. The rice YAC library contains an average DNA insert of 350 kb, which may be extremely valuable for constructing large DNA contigs over a particular genomic region or whole chromosomes. However, 40% of clones in the library are chimeric (Umehara *et al.*, 1995), requiring that 40% more clones need to be screened to locate the desired sequence. In contrast we have found no evidence for chimerism in the rice BAC library (Jiang, Gill, Wang, Ronald and Ward, unpublished results). In addition, since preparation, screening and manipulation of BAC clones is much more efficient than with YAC clones, we have easily identified 12 clones linked to *Xa-21* locus. This library can

also be used to isolate BACs containing other genes of interest and their corresponding cDNAs. In the future, it may be possible to transfer directly large inserts of DNA into rice using *Agrobacterium*-mediated transformation and a modified BAC vector containing T-DNA borders (Hiei *et al.*, 1994; Lloyd, Stanford University, personal communication).

Compared with rice, other cereal crops such as maize, wheat, barley and rye have a much higher percentage of repetitive sequences, which hinder the application of chromosome walking strategies for isolation of important genes. Moore *et al.* (1993) reported that 60% of the single-copy rice DNA sequences which cross-hybridized to wheat and barley DNA detected single-copy sequences in these genomes. Most of the RFLP markers on wheat chromosome linkage group 5 showed the same linkage ordering between rice and wheat (Kurata *et al.*, 1994). Single-copy end probes of rice BAC clones could be used to jump or walk between genes in wheat to avoid the repetitive sequence. Thus, 'inter-genomic cloning' (M.D. Gale, UK) will be another important application of the rice BAC library.

A 6× genome-equivalent cosmid library was constructed in our lab from the same rice material that was used in the BAC library construction (unpublished data). No cosmid clones were identified carrying the *pta818* sequence. In contrast, five BACs out of 7296 BAC clones tested hybridized with this sequence. This result suggests that the BAC system may allow cloning of DNA fragments that are not present in cosmid libraries.

We have successfully used this BAC cloning procedure in other species such as cowpea, *Arabidopsis*, lettuce and tomato (unpublished results). Recently, a sorghum BAC library has also been constructed (Wing, Texas A&M, personal communication). Since BAC libraries can be constructed in a short time and analyzed more efficiently than YAC or P1 libraries, the BAC system is likely to become an important tool for map-based cloning of genes from crop species.

Experimental procedures

Preparation of HMW DNA in rice

An IRRI (International Rice Research Institute) rice line, IR-BB21 carrying *Xa-21* was used as the plant material in this study. The plants were grown in the greenhouse for 3–5 weeks. Leaf tissue was harvested and washed with distilled water before grinding. HMW DNA was extracted from rice tissue essentially as described by Hatano *et al.* (1992) and Zhang *et al.* (1994), with the following modifications: approximately 20 g of leaf tissue were ground into powder using a cold mortar and pestle in liquid nitrogen. The powder was suspended by stirring in 200 ml cold nuclei-extraction (NE) buffer (1 mM spermidine, 1 mM spermine, 10 mM Na₂ EDTA, 10 mM Trizma base, 80 mM KCl, 0.5% Triton-X 100 and 0.4 M sucrose, pH 9.4). The mixture was filtered through two layers of cheesecloth into a GSA bottle and centrifuged at 1200 g at 4°C for

20 min. The supernatant was poured off and the nuclear pellet (pale green) was resuspended in 50 ml cold NE buffer. The resuspended pellet was then filtered through an 80 µm sieve into a 50 ml tube to remove green tissue debris and then centrifuged at 1000 g for 10 min. The pellet was resuspended and centrifuged as above without passing through the 80 µm sieves. The nuclear pellet (about 5×10^8 nuclei ml⁻¹) was resuspended in 2.5 ml of SCE buffer (1 M sorbitol, 0.1 M sodium citrate, 60 mM EDTA, pH 7.0) and embedded in 2.5 ml 1% low-melting-point (LMP) agarose (Ultrapure). Plugs (80 µl) were incubated in 25 ml ESP solution (0.5 M EDTA, pH 9.3, 1% sodium lauryl sarcosine, 5 mg ml⁻¹ proteinase K, Boehringer Mannheim) at 50°C for two days with one change of the buffer. Each plug contained about 5 µg DNA.

Partial digestion of HMW DNA and size fractionation by PFGE

Agarose plugs were dialyzed twice against TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) plus 1 mM PMSF (phenylmethyl sulfonyl fluoride) at 50°C for 1 h, and then equilibrated with *Hind*III buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂ and 1 mM dithiothreitol, pH 7.9) twice at room temperature for 1 h. Plugs were melted at 65°C for 15 min and kept at 37°C for 5 min before partial digestion. Five to seven units of *Hind*III (NEB, USA) per plug were added to the DNA solution and incubated at 37°C for 30 min. The reaction was stopped by addition of 1/10 volume of 0.5 M EDTA, pH 8.0. Partially digested DNA was immediately loaded into a 0.8% LMP agarose gel with a pipette tip cut off to an inside diameter of 2 mm and separated by PFGE (CHEF DR II system, Bio Rad, USA). Two different PFGE methods were used for the library construction. Firstly, the gel was subjected to electrophoresis at 150 V, using an 8 sec initial and 8 sec final switch time for 16 h at 14°C. The unresolved DNA (≥ 200 kb) was focused into a thin band. Secondly, the gel was subject to electrophoresis at 150 V, ramped switching time from 60 to 90 sec for 16 h at 14°C. For both methods, the gel containing the partially digested DNA was cut and soaked in TE while the marker lanes of the gel were stained with ethidium bromide. The agarose slice containing fragments larger than 200 kb (the first PFGE method) or agarose slice containing 250–350 kb (the second method) was excised from the gel. The agarose slice was equilibrated in TE for 2 h at 4°C, placed in a 1.5 ml tube, melted at 65°C for 10 min, digested with Gelase (Epicentre, USA) (one unit of enzyme per 100 mg agarose) and incubated at 45°C for 1 h. The DNA solution was directly used for the ligation reaction.

Isolation and preparation of vector, and ligation reaction

The vector, pBeloBAC II, was kindly provided by Drs H. Shizuya and M. Simon (California Institute of Technology, USA). This vector contains the *lacZ* gene inserted into the vector pBAC108L (Shizuya *et al.*, 1992). A single colony was inoculated into 5 ml LB media containing 12.5 µg ml⁻¹ chloramphenicol and grown at 37°C for 4–5 h before adding to 6 l of LB media. The inoculum was grown for about 16 h at 37°C to an OD_{600nm} 1.3–1.5. The plasmid was isolated using Qiagen's plasmid Maxi isolation kit (Qiagen, USA). Vector DNA was further purified by cesium chloride/ethidium bromide equilibrium centrifugation at 45 000 r.p.m. for 60 h. The rotor was decelerated to 35 000 r.p.m. for 1 h to allow the gradient to relax, using a fixed-angle rotor 70.1 (Beckman, USA). The plasmid was digested with *Hind*III to completion and assayed by gel electrophoresis. Vector ends were dephosphorylated with HK

phosphatase (Epicenter, USA) at 30°C for 1 h, using 1 unit of the enzyme per 1 mg of vector DNA. The HK phosphatase was inactivated by heating at 65°C for 30 min. The ligation was carried out in a 100 ml volume in which about 40 ng of the size-selected rice DNA (about 85 μ l) was ligated to 10 ng of *Hind*III-digested vector (1 μ l) (molar ratio of about 10:1, with vector excess) with 400 units of T4 DNA ligase (NEB, USA) at 16°C for overnight. Before transformation, the ligation was dialyzed against TE in an ULTRAFREE-MC filter tube (Millipore, USA) at 4°C overnight.

BAC transformation

Transformation of competent *Escherichia coli* DH10B cells (Gibco-BRL, USA) was carried out by electroporation using a Cell-Porator (Gibco-BRL, USA) at the following settings: voltage, 400; charge rate, fast; voltage booster resistance, 4000; capacitance, 330 μ F; impedance, low. Thirteen microliters of competent cells were mixed with 0.5–1.0 μ l of ligation solution for each electroporation. After electroporation, cells were transferred to 1 ml SOC solution (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose, pH 7.0) and incubated at 37°C with gentle shaking (90–95 r.p.m.) for 45 min. The cells were spread on LB plates containing chloramphenicol (12.5 μ g ml⁻¹), X-gal (40 μ g ml⁻¹) and IPTG (isopropylthio- β -D-galactoside) (0.072 μ g ml⁻¹). Plates were incubated at 37°C for 24 h. White colonies containing rice DNA inserts were picked to a new LB plate for a second color screen. The BAC clones were transferred to 384 well microtiter plates (Genetix, UK) containing 60 μ l of LB freezing buffer (36 mM K₂HPO₄, 13.2 mM KH₂PO₄, 1.7 mM citrate, 0.4 mM MgSO₄, 6.8 mM (NH₄)₂SO₄, 4.4% v/v glycerol, 12.5 μ g ml⁻¹ chloramphenicol, LB) and incubated at 37°C for 24 h. Since more than 95% of the colonies were still white on the second screen, only one screen was used in the subsequent experiments, and white colonies were directly picked to 384-well microtiter plates. The library was replicated in duplicate and stored in two different -80°C freezers.

Filter preparation and screening

The BAC clones in each 384 well microtiter plate were replicated on to a Hybond N⁺ filter (Amersham, USA). The filter was put into a plastic box containing LB/agar with 12.5 μ g ml⁻¹ chloramphenicol and the box was kept at 37°C overnight until the colonies were about 2–3 mm in diameter. Treatment of the filters was as described (Hoheisel *et al.*, 1993; Nizectic *et al.*, 1990). Hybridization and washing conditions were the same as described in Hoheisel *et al.*, (1993). Probes were labeled using random primer extension (Feinberg and Vogelstein, 1983).

DNA probes

The four rice repeated sequences (*pOs48*, *pOa4*, *pOo2* and *pOb1*) were provided by Dr R. Wu (Cornell University, USA). The maize chloroplast *RBCL* gene and mitochondria *COI* gene were from Dr D. Stern (Boyce Thompson Institute, USA). Rice chloroplast clones (P1 and P4) were provided by Dr T. Ishii (Kobe University, Japan). Telomere synthesized sequence, (TTTAGGG)₁₀, was provided by Dr K. S. Wu (Du Pont, USA).

Isolation of BAC DNA and determination of rice DNA insert size of BAC clones

BAC clones were inoculated into 1.5 ml LB (containing 12.5 μ g ml⁻¹ chloramphenicol) and incubated with shaking for 24 h.

The alkaline lysis method (Sambrook *et al.*, 1989) was used for preparing the BAC DNA. One phenol/chloroform purification was performed before ethanol precipitation. The BAC DNA was digested with *Not*I (Gibco-BRL, USA) for 3 h before loading on a 1.0% agarose gel. The gel was subjected to PFGE electrophoresis at 150 V, using a 10 sec switch time for 16 h at 14°C. The size of each BAC clone was determined by comparing with a λ concatamer size standard run in the same gel.

Southern blot analysis of BAC clones using total genomic DNA and DNA markers

Gel transfer to Hybond-N⁺, hybridization and washing conditions were performed according to manufacturer's instructions (Amersham, USA).

Stability tests of rice BACs

Two BAC clones containing 140 kb and 210 kb DNA inserts were inoculated into 500 ml LB and grown at 37°C overnight. The cultures were diluted 5 \times 10⁵ times each day to inoculate fresh 500 ml LB for overnight growth. This process was repeated for 5 consecutive days. DNA isolated from day 1 and day 5 were digested with *Xho*I, *Not*I, *Sfi*I, *Hind*III and electrophoresed using PFGE to check their restriction patterns.

Effect of BAC DNA insert size on E. coli transformation efficiency

Seven BAC clones of different sizes (40–220 kb) and the pBeloBAC II vector (7.0 kb) were used in the experiment. The DNA concentrations of the BAC clones and the vector were determined using a DNA fluorometer. Electroporation was performed by adding 10 μ g, 100 μ g or 1 ng of DNA to 13 μ l of competent cells (DH10B). The transformation procedures were the same as described above. The transformation efficiencies were calculated as the number of transformants per femtomole of BAC DNA. The number of molecules per microgram of DNA were calculated based on insert sizes plus the vector.

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