Construction and Characterization of a Bacterial Artificial Chromosome Library of the *Indica* Rice Kasalath

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Summary

We constructed a bacterial artificial chromosome (BAC) library for the *indica* rice variety Kasalath using a new BAC vector, pCHR8, suited for plasmid rescue, containing nine unique restriction sites in a multicloning site. This Kasalath BAC library consists of about 65,000 clones with an average insert size of 106 kb using *Hind*III partially digested DNA fragments. It represents approximately sixteen rice haploid genome equivalents which are sufficient for the full coverage of the rice genome with BAC clone contigs. The library was constructed from only a single ligation mixture with high transformation efficiency. The quality of this BAC library was ascertained by successfully retrieving the BAC clones for seven rice RFLP markers. We could isolate eleven individual BAC clones using RFLP markers that linked to the lethal factor locus for segregation distortion on rice chromosome 3. The content of the chloroplast and mitochondrial DNA in this library was estimated to be 2.8% and 0.4%, respectively. These results demonstrate that this Kasalath BAC library should be suitable for map-based cloning and physical mapping of the rice genome.

Key Words : *Oryza sativa* L., bacterial artificial chromosome (BAC) library, BAC vector, map-based cloning, plasmid rescue, segregation distortion.

Introduction

The bacterial artificial chromosome (BAC) cloning system has provided a powerful tool for physical mapping and map-based cloning in genome research. This cloning system utilizes the *Escherichia coli* (*E. coli*) F plasmid for its replicating system and has a cloning capacity of exogenous DNA fragments as large as 300 kb (Shizuya *et al.* 1992). The BAC plasmid vector's replicating system essentially consists of two functional units, the F plasmid replication origin and the partition locus, the F plasmid replication origin drives its copy number at one to two copies per *E. coli* chro-

mosome, and the partition locus ensures its maintenance during cell division (Willets and Skurray 1987).

Though yeast artificial chromosome (YAC) libraries of large insert sizes may have some value for the framework of the genome research, especially in a large complex genome, the BAC library system has advantages over the YAC system; high transformation efficiency, very low level of chimerism of clones, easy manipulation of BAC plasmid DNA from host *E. coli* cells and clone stability for long term maintenance (Shizuya *et al.* 1992, Woo *et al.* 1994). The BAC library and high-density molecular linkage map provide the access to the target gene by chromosome landing or chromosome walking (Tanksley *et al.* 1995).

The construction of BAC libraries has been reported during the development of plant genome research; such as sorghum (Woo *et al.* 1994), rice (Tao *et al.* 1994, Wang *et al.* 1995, Zhang *et al.* 1996, Nakamura *et al.* 1997, Yang *et al.* 1997), *Arabidopsis* (Choi *et al.* 1995, Mozo *et al.* 1998) and soybean (Marek and Shoemaker 1997, Danesh *et al.* 1998). For *Arabidopsis* genome research (Meinke *et al.* 1998), BAC libraries were used to construct the sequence-ready map and were suited to minimize the gaps for the physical mapping based on YAC contigs. Furthermore, BAC clones can be directly used as substrates for DNA shotgun sequencing.

Rice is a model crop among cereals for genome research, because of its small genome size (Arumuganathan and Earle 1991) and availability of a large number of DNA markers on the genetic linkage maps (Saito *et al.* 1991, Causse *et al.* 1994, Harushima *et al.* 1998). Moreover, the synteny of the major gene order between rice and other cereals should provide for the isolation of biologically important genes in other cereals by map-based cloning (Gale and Devos 1998). The sequencing projects of the rice genome have been initiated internationally. Rice BAC and YAC clones have been used for the construction of the physical mapping of the whole genome (Hong *et al.* 1997, Kurata *et al.* 1997, Zhang and Wing 1997). The resistance gene to bacterial pathogen in rice has been isolated and characterized by map-based cloning with the aid of BAC clones (Song *et al.* 1995).

The *indica* rice Kasalath is a widely used variety in the molecular genetics of rice. Using F₂ populations from crosses between *indica* variety Kasalath and *japonica* varieties, FL134 and Nipponbare, extensive rice molecular linkage maps have been constructed (Saito *et*

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al. 1991, Harushima *et al.* 1998). Furthermore, agronomically important quantitative trait loci (QTL) were analyzed in detail using Kasalath as one of the cross parents (Yano and Sasaki 1997). Because the information about a large number of rice DNA markers and the map position of agronomically important genes and QTLs are available, it should be expected that Kasalath BAC library is an important resource for BAC contig formation and map-based cloning.

In this paper, we report the construction of about 65,000 clones of a BAC library of the *indica* rice variety Kasalath. This BAC library can be used to isolate genomic sequences in the region of the lethal factor locus on rice chromosome 3. We also describe the utility of BAC vector pCHR8 for chromosome walking and map-based cloning.

Materials and Methods

Plasmid

The pBeloBAC11 vector (Kim *et al.* 1996, Wang *et al.* 1997) was kindly provided by Dr. R. A. Wing (Texas A & M University, College Station, TX, USA, present address: Clemson University, Clemson, SC, USA). pNEB193 was purchased from New England Biolabs, USA. All plasmid vectors were transformed by electroporation using BRL Cell-Porator system (BRL, USA) to *E. coli* ElectroMAX DH10B cells (BRL, USA) according to the protocol provided by the manufacturer.

Construction of BAC cloning vector, pCHR8

To introduce the *Swa*I site to pNEB193, two oligonucleotides were designed, 5'-GATTTAAAT-3' and 5'-ATTTAAATC-3' (underline: *Swa*I site). This *Swa*I linker was inserted in the *Hinc*II site at the multicloning site of pNEB193 to produce pCHR1. This 9 bp insertion does not disturb the coding frame of *lacZ* for α -complementation. To engineer the upstream region of the *Hind*III site of pBeloBAC11, *Nhe*I and *Mlu*I sites were constructed using two synthetic oligonucleotides, 5'-AGCTTGGGCTAGCTACGCGTG-3' and 5'-AGCTCAGCGTAGCTAGCCCA-3' (underline: *Nhe*I and *Mlu*I sites). This polylinker was then inserted in the *Hind*III site of pBeloBAC11 in the correct orientation to produce pCHR5, so that pCHR5 has a fully functional α -complementation activity. To construct pCHR8, 0.09 kb of *Eco*RI and a *Hind*III fragment of pCHR1 was ligated to 7.47 kb of *Eco*RI and a *Hind*III fragment of pCHR5.

BAC vector preparation

A BAC cloning vector, pCHR8, was isolated from a 4-liter overnight culture by the alkaline lysis method as described by Sambrook *et al.* (1989). Plasmid DNA was further purified by cesium chloride density gradient centrifugation. The pCHR8 vector was digested with *Hind*III, followed by dephosphorylation with calf intestine alkaline phosphatase (Takara, Japan). The effi-

ciency of dephosphorylation was estimated by counting the colonies produced from the self-ligation of dephosphorylated BAC vector and non-dephosphorylated BAC vector. The preparations in which dephosphorylation resulted in more than 95% reduction in the number of transformants were used for library construction.

Plant material

Mature seeds of *indica* rice variety Kasalath were sterilized with 70% ethanol for one min and 10 % sodium hypochlorite solution (Wako, Japan) for 15 min. The seeds were washed with water for 30 min and allowed to germinate in a growth chamber at 25 °C in the dark. For the preparation of rice high molecular weight DNA, the etiolated leaf tissues of 12-14 day old were harvested, frozen in liquid nitrogen and stored at -80 °C.

Preparation of high molecular weight DNA

Nuclei were isolated from rice etiolated leaf tissues as described by Zhang *et al.* (1995). The final nuclei pellet (0.8 ml) prepared from 70 g of fresh weight tissues was embedded in an equal volume of 1 % SeaPlaque GTG agarose solution (FMC, USA). The mixture was then poured into ice-cold plug molds (Bio-Rad, USA) at 80 μ l per plug and stored at 4 °C for 30 min. After the agarose was solidified completely, the plugs were incubated in 0.5 M Na₂EDTA (pH 9.0), 1% sodium lauryl sarcosine and 0.1 mg/ml proteinase K (Boehringer Mannheim, Germany) at 50 °C for 2 days with one change of the same lysis buffer. To inactivate the proteinase K, the plugs were then washed once with 0.5 M Na₂EDTA (pH 9.0) for 1h and three times with TE (10 mM Tris-HCl, pH 8.0, 1 mM Na₂EDTA) containing 0.1 mM phenylmethyl sulfonyl fluoride for 1h at 4 °C. Finally, the plugs were washed three times in TE for 1h at 4 °C and stored at 4 °C until used.

*Hind*III partially digested rice high molecular weight DNA

Plugs containing rice nuclei were incubated twice with *Hind*III reaction buffer (Woo *et al.* 1994) for 1 hr at room temperature. The plugs were then melted at 65 °C for 15 min and held at 37 °C for 5 min. For each plug (80 μ l) containing about 40 μ g DNA, 0.2 units of *Hind*III (Takara, Japan) was added and incubated for a further 30 min at 37 °C. The reaction was stopped by the addition of one tenth volume of 0.5 M Na₂EDTA (pH 8.0).

Partially digested rice DNA was separated by two rounds of size fractioning (Woo *et al.* 1994, Asakawa *et al.* 1997) performed on a pulsed field gel electrophoresis using a CHEF (contour clamped homogeneous electric field) Mapper (Bio-Rad, USA). For the first size selection, the conditions were; 1% SeaPlaque GTG (low melting point) agarose gel in 1 $\times$ TAE buffer (Sambrook *et al.* 1989), at 6 V/cm, with a linearly ramped pulse time of 0.22 s to 93.7 s, for 4 h at 11 °C. After removal of

the gel portion containing fragments smaller than 100 kb and the rest of the agarose gel was replaced in the sample trough of a new 1% SeaPlaque GTG agarose, followed by pulsed field gel electrophoresis under the same conditions except for a running time of 11h. The region containing the rice DNA fragments from 150 to 300 kb in size was excised from the gel and was again replaced in the trough in a new 1% SeaPlaque GTG agarose gel for the second size selection. The conditions for the second size selection were in $1 \times$ TAE buffer, at 6 V/cm, with a linearly ramped pulse time of 0.22 s to 3.4 s, for 15h at 11 °C. The compressed portion of DNA in the gel was excised from the gel and stored at 4 °C. Lambda ladder PFG marker and Mid Range II PFG marker (New England Biolabs, USA) were used as the molecular weight standards.

Construction of the BAC library

A gel piece containing size-selected DNA was melted at 65 °C for 15 min and digested with 1 unit of GELase (Epicentre, USA) per 100 mg of gel at 45 °C for 1 h. The size-selected DNA solution was transferred onto 0.025 μ m filters (VSWP 02500, Millipore, USA) floating on 30 ml of dialysis buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM Na₂EDTA) in a petri dish and spot-dialyzed for 30 min at room temperature. The concentration of dialyzed DNA was estimated by comparing the intensity of ethidium bromide stain under ultraviolet with standards of known concentration of lambda DNA (Takara, Japan). Forty ng of size-selected DNA was ligated to 5 ng of dephosphorylated pCHR8 in a molar ratio of 1 (rice DNA) to 2.5 (vector) in a total volume of 50 μ l with 0.5 units of T4 DNA ligase (Nippon Gene, Japan) in 66 mM Tris-HCl (pH 7.6), 6.6 mM MgCl₂, 10 mM DTT and 0.1 mM ATP. The mixture was incubated at 16 °C for 24 h and stored at 4 °C until used.

Each 0.5 μ l of ligation solution was transformed to 13 μ l of *E. coli* ElectroMAX DH10B cells using a BRL Cell-Porator system. The setting conditions are voltage, 330; capacitance, 330 μ F; impedance, low ohms; charge rate, fast; Voltage Booster resistance, 4000 ohms. After electroporation, 1 ml of SOC media (Sambrook *et al.* 1989) was added to the cuvette, transferred to a 15-ml sterile test tube, and incubated at 37 °C with shaking at 150 r.p.m. for 1h. The culture was spread on an LB agar plate containing 12.5 μ g/ml Cm and 240 μ l 2% X-gal and 20 μ l of 100 mM IPTG were spread on one 150-mm diameter plate. After 36 h of growth at 37 °C, white (recombinant) and blue (nonrecombinant) color colonies were clearly identified. White colonies were picked up with a sterile toothpick and transferred to an individual well of a 96-well microtiter plate (Nalge Nunc, USA), containing 100 μ l of LB freezing buffer (Woo *et al.* 1994). Microtiter plates were incubated at 37 °C for 18h, and stored at -80 °C.

DNA markers

Rice RFLP markers, XNpb15, XNpb23, XNpb44,

XNpb132, XNpb187, XNpb342 and XNpb394, originate from a rice genomic *Pst*I library as described by Saito *et al.* (1991). The three rice chloroplast DNA fragments (Hiratsuka *et al.* 1989); a *Bam*HI 5.0 kb fragment (*Bam*7) for the *psbA* gene, a *Pst*I 10.9 kb fragment (*Pst*5) for the *ndhA* gene and a *Pst*I 0.3 kb fragment (*Pst*15) for the *rbcl* gene and six mitochondrial DNA fragments (Iwahashi *et al.* 1992); an *Sal*I 7.0 kb fragment for the *atp9* gene, an *Sal*I 6.4 kb fragment for the *atpA* gene, an *Sal*I 7.5 kb fragment for the *coxI* gene, an *Sal*I 1.9 kb fragment for the *coxII* gene, an *Eco*RI 0.7 kb fragment for the *nad3* gene and an *Eco*RI 1.4 kb fragment for the *cob* gene were kindly provided by Dr. A. Hirai (The University of Tokyo, Japan).

Southern blot analysis

BAC plasmid DNA was isolated from 5-ml cultures grown overnight in LB containing 12.5 μ g/ml Cm using an automated plasmid miniprep machine (Kurabo Model PI 50, Japan). The purified plasmid DNA (about 500 ng) was digested with 5 units of *Not*I at 37 °C for 3 h. *Not*I-digested BAC DNA was subjected to pulsed field gel electrophoresis using a CHEF Mapper. The conditions were; 1% pulsed field certified agarose (Bio-Rad, USA) gel in 0.5 $\times$ TBE buffer (Sambrook *et al.* 1989) with a linearly ramped pulse time of 0.22 s to 12.2 s, for 15 h at 11 °C. The DNA was transferred from gel to Hybond N+ membrane according to the manufacturer (Amersham, USA). Prehybridization and hybridization conditions were the same as described by Sambrook *et al.* (1989). Probes were labeled with [α -³²P] dCTP using a Multiprime DNA labelling system (Amersham, UK).

High density colony filter preparation

The 3072 BAC clones from 32 stored 96-well microtiter plates were blotted on Hybond-N+ membrane placed on LB agar plate containing 12.5 μ g/ml Cm in Nunc Omuni tray, in a 6 $\times$ 6 grid pattern using a 96-pin replicator of Biomek 2000 workstation (Beckman, USA). For negative positional markers, the 7th, 10th, 25th and 28th positions of 6 $\times$ 6 grid were blotted with LB containing Cm. The plates with BAC clones were incubated at 37 °C for approximately 18 h. Colony hybridization was performed as described by Zhang *et al.* (1996).

Plasmid rescue

To isolate the right and left end fragments of *Hind*III inserted BAC clones, about 50 ng of the purified BAC DNA was digested with *Bam*HI or *Sph*I for the right side and *Mlu*I or *Nhe*I for the left side for 3 h at 37 °C. The digests were self-ligated at 16 °C overnight, and the resultant ligates were transformed into ElectroMAX DH10B cells by electroporation. Cm resistant transformants were further analyzed.

Fig.1. Structure of a newly constructed BAC vector, pCHR8 and the nucleotide sequence of the multicloning site. Unique restriction sites are shown by italics. *lacZ'* β -galactosidase gene for α -complementation, *cat* chloramphenicol acetyltransferase gene, *oriF* essential region for replication of F plasmid, *par* partition locus of F plasmid, *cosN* bacteriophage λ terminase recognition site, *loxP* bacteriophage P1 Cre recombinase recognition site. The DDBJ accession number for the total sequence of pCHR8 is AB015619.

Results

Construction of a new BAC vector, pCHR8, containing nine unique sites in a multicloning site

We constructed a new BAC vector, pCHR8 (Fig. 1). This vector is a derivative of pBeloBAC11 (Kim *et al.* 1996) with the modified multicloning site. The total nucleotide length of pCHR8 is 7564 bp. The multicloning site of pCHR8 contains four unique eight-base recognized restriction sites, *AscI*, *PacI*, *SwaI* and *PmeI*, and five unique six-base recognized restriction sites, *BamHI*, *SphI*, *HindIII*, *NheI* and *MluI*. These changes introduced in the multicloning site showed no detectable effect on the function of *lacZ*-based colony selection. BAC cloning vector pCHR8 can propagate and remain stable in *E. coli* DH10B cells in LB media containing 12.5 μ g/ml Cm.

Plasmid rescue is a simple and precise method to obtain the end fragments from BAC clones. The *HindIII* site is the most frequently used cloning site for the construction of a BAC library. As shown in Fig. 1, BAC vector pCHR8 has four unique six-base recognized restriction sites up to *NotI* site, *SphI*, *BamHI*, *NruI* and *ApaLI* for the isolation of right end fragments of *HindIII* inserted BAC clones. However, in previous BAC vectors such as pBeloBAC11 and pECSBAC4 (Frijters *et*

al. 1997), convenient restriction enzyme sites are not present on the right side of *HindIII* site. For this reason, we introduced unique *NheI* and *MluI* sites on the right side of *HindIII* site of pCHR8 for the isolation of left end fragments by plasmid rescue.

Construction of a BAC library of indica rice Kasalath

We have constructed an *indica* rice BAC library consisting of 65,000 clones. The clones were produced by partial *HindIII* digestion of high molecular weight DNA from etiolated *indica* rice seedlings. This BAC library was constructed using a single ligation mixture of 50 μ l. Over 90 % of the chloramphenicol-resistance clones on the plates were white colonies. Approximately 1400 recombinant clones were obtained per μ l of ligation product. The cloning efficiency was about 1.4×10^7 clones per μ g plasmid DNA. This efficiency did not show an evident decline for three weeks after ligation reaction. We completed all procedures from ligation reaction to freezing stock of 65,000 BAC clones with two months.

The insert sizes were determined by digestion of randomly selected white colonies with *NotI* digestion followed by pulse field gel electrophoresis (Fig. 2). All of one hundred eight BAC clones were contained in the insert DNAs ranging from 50–265 kb. Fig. 3 illustrates the size distribution of BAC clones. We estimate the

Fig.2. Ethidium bromide-stained agarose gel showing 43 random rice Kasalath BAC clones. Isolated BAC DNAs were digested with *NotI* and separated by a CHEF Mapper. Arrow indicates the 6.9 kb *NotI* digested pCHR8 vector band. Lane M is a Mid Range II PEG marker.

Fig.3. Insert size distribution of *indica* rice Kasalath BAC library.

average insert size of BAC clones in this library to be 106 kb. Assuming a haploid genome size of 4.3×10^8 bp for rice (Arumuganathan and Earle 1991), the library represents approximately sixteen genome equivalents. This result indicates that the Kasalath BAC library has 99.9 % probability of completing the continuous overlapping contig in the rice genome according to the formula as described in Zhang and Wing (1997).

BAC library screening with rice RFLP markers and identification of BAC clones linked to the lethal factor locus on rice chromosome 3

To estimate the ability to isolate a specific DNA clone from this library, we constructed the high density colony filters screenable by colony hybridization. BAC clones were arrayed onto microplate-sized membranes as high

-density matrix replicas. Each replica filter contains 3072 BAC clones, arrayed as a 6×6 mini matrix with four positional markers. We prepared six high-density colony filters containing a total of $6 \times 3072 = 18,432$ BAC clones. This filter set corresponds to 28 % of the total BAC clones in this library and 4.5 rice haploid genome equivalents. Seven of single-copy rice RFLP markers were used for BAC library screening with colony hybridization. RFLP markers XNpb132, XNpb342, XNpb187 and XNpb44 are located on chromosomes 2, 6, 8 and 11. XNpb15, XNpb23 and XNpb394 are tightly linked to the lethal factor locus on the middle of chromosome 3 (Cheng *et al.* 1996).

Table 1 summarizes the results of the library screening with rice RFLP markers. To confirm positive signals, we isolated BAC DNAs from hybridizing clones, digested with *NotI*, fractionated on CHEF gel and analyzed by Southern hybridization. For each of these RFLP markers, at least two corresponding BAC clones were identified from 18,432 BAC clones. As a result, we estimate that at least seven independent BAC clones could be recovered from a total of 65,000 BAC clones. This value is sufficient for the coverage of the rice genome by BAC clones. We could also obtain the BAC clones hybridizing to RFLP markers XNpb23 and XNpb15 tightly linked to the lethal factor locus. Fig. 4 shows ethidium bromide-stained agarose gels and auto-

Fig.4. Analysis of BAC clones hybridizing to RFLP markers, XNpb23 (A) and XNpb15 (B). (A) The left panel shows ethidium bromide-stained agarose gel containing six BAC clones hybridizing to XNpb23. BAC DNAs were digested with *NotI* and separated by a CHEF Mapper. The right panel shows a Southern blot of the same gel hybridized with XNpb23 probe. (B) The left panel shows an ethidium bromide-stained agarose gel containing five BAC clones hybridizing to XNpb15. BAC DNAs were digested with *NotI* and separated by a CHEF Mapper. The right panel shows a Southern blot of the same gel hybridized with XNpb15 probe. Names of the individual BAC clones are listed above the lanes. Arrow indicates the 6.9 kb *NotI* digested pCHR8 vector band. Lane M is a Mid Range II PEG marker.

Table 1. BAC clones screened by rice RFLP markers

RFLP marker	Rice chromosome	BAC clone name	Insert size (kb)
XNpb132	2	K131C8	125
		K159C3	105
XNpb23	3	K19B1	105
		K37C3	95
		K63D7	100
		K106C7	125
		K135C8	90
XNpb15	3	K18E3	90
		K39G1	85
		K119C4	105
		K121E11	170
		K122A6	120
		K132H9	90
XNpb394	3	K75B7	75
		K606F5	95
		K615A6	90
XNpb342	6	K25E1	175
		K63D1	75
		K81F5	110
		K88D9	145
		K112H1	75
		K160C1	250
		K629A11	175
XNpb187	8	K18A6	125
		K35G11	110
		K48H8	125
		K61A11	120
		K86D2	125
		K106A4	110
		K125A11	165
		K159C7	135
XNpb44	11	K38H6	260
		K41G3	90
		K42D4	135
		K50H7	135
		K69C2	255
		K75F1	85
		K154C6	70
		K619D5	80
		K626G11	115
No. of total clones		40	
No. of average hits per RFLP marker		5.7	
Average of inserts (kb)			122

radiograms of XNpb23 and XNpb15 hybridizing five and six BAC clones. In both cases, some common bands were observed with *NotI* digestion. This indicates that BAC clones with common bands were derived from the same position in the rice genome.

Screening the library for chloroplast and mitochondrial sequences

Fig.5. The strategy for the isolation of left and right end clones from BAC insert DNA with plasmid rescue. Hatched box indicates *MluI*- or *NheI*- digested left end DNA of BAC insert. Dotted box indicates *SphI*- or *BamHI*- digested right end DNA of BAC insert.

Fig.6. Ethidium bromide-stained agarose gel showing right (A) and left (B) end fragments of BAC end clones by plasmid rescue. (A) *SphI* and *HindIII* digestion of BAC right end clones. (B) *MluI* and *HindIII* digestion of BAC left end clones. Arrow indicates the 7.6 kb fragment of pCHR8 vector. Lane M is a *HindIII*- digested λ DNA marker.

Chloroplast and mitochondrial DNA contents were estimated by hybridizing the same high-density filters with three rice chloroplast probes and six rice mitochondrial probes. Three fragments of the rice chloroplast DNA are from 15 to 60 kb apart (Hiratsuka *et al.* 1989). Six fragments of the rice mitochondrial DNA are separated by 10 to 210 kb on the basis of a master circle of the rice mitochondrial genome (Iwahashi *et al.* 1992). Of 18,432 BAC clones, we identified 512 hybridizing clones to three chloroplast probes and 80 hybridizing clones to six mitochondrial probes. According to these estimates, 2.8 % of BAC clones carried chloroplast DNA and 0.4 % of BAC clones carried mitochondrial

DNA. This indicates that the degree of contamination of chloroplast and mitochondrial DNA is very low and that this Kasalath BAC library is well suited for map-based cloning and physical mapping of the rice genome.

Isolation of right and left ends of BAC clones by plasmid rescue

Plasmid rescue has been used to isolate the right and left ends of BAC clones. Fig. 5 shows the strategy for the isolation of right and left ends of *Hind*III inserts of pCHR8-based BAC clones. To isolate the right ends of BAC clones, we used *Sph*I or *Bam*HI to digest BAC DNAs, and the resulting fragments were self-ligated, followed by transformation into *E. coli* DH10B cells. Plasmid DNAs of the transformants were isolated and verified by digestion with *Sph*I or *Bam*HI and *Hind*III. Fig. 6A shows *Sph*I- and *Hind*III-digested products of the right ends derived from three BAC clones, K122A6, K63D7 and K606F5, hybridizable to RFLP markers XNpb15, XNpb23 and XNpb394, respectively. The right end clone of K606F5 gave two fragments (Fig. 6A), because this end clone has one internal *Hind*III site between the *Hind*III site used for BAC cloning and the outermost *Sph*I site.

To isolate the left ends of BAC clones, BAC DNAs were digested with *Nhe*I or *Mlu*I, self-ligated and transformed in *E. coli* cells (Fig 5). Fig. 6B shows the *Mlu*I- and *Hind*III-digested products of the left ends derived from the same BAC clones in Fig. 6A. The end clones of K122A6 and K63D7 have one and two internal *Hind*III sites, respectively; the digestion of *Mlu*I and *Hind*III gave two and three fragments (Fig. 6B). Using this procedure, we could isolate the end fragments of BAC clones ranging from 0.2 kb to 20 kb.

Discussion

We constructed a new BAC vector, pCHR8, capable of identifying the inserts by *lacZ*-based color selection. The multicloning site of pCHR8 contains four unique eight-base recognized restriction sites and five unique six-base recognized restriction sites. The introduced eight-base recognized restriction sites can facilitate the restriction mapping of large insert BAC DNAs by pulse field gel electrophoresis. Particularly, the *Mlu*I site might be useful for map-based cloning on complex large genomes, because Moore *et al.* (1993) indicated that the unmethylated *Mlu*I site is distributed nonrandomly in the cereal genomes and might be associated with gene islands. The *Mlu*I cloning site of pCHR8 would be applicable to the construction of BAC libraries containing gene islands with high frequency. To facilitate chromosome walking, it is important to recover the end fragments of BAC clones used as probes to rescreen the library. Though PCR procedures such as Vectorette-PCR and TAIL-PCR are powerful for the isolation of BAC end fragments, they sometimes encounter non-efficient amplification. On the other hand, plasmid

rescue requires some extra steps compared with PCR procedures, but it is a precise procedure for the end fragment isolation. The *Hind*III inserted clones of pCHR8 permit the isolation of both right and left end fragments by plasmid rescue.

At present, six rice BAC libraries have been reported with average insert sizes of 107 to 155 kb (Tao *et al.* 1994, Wang *et al.* 1995, Zhang *et al.* 1996, Nakamura *et al.* 1997, Yang *et al.* 1997). However, the maximum library size was seven rice haploid genome equivalents. It is still necessary to increase the library size for the full coverage of the rice genome with BAC clones. In this report, we constructed a BAC library of the *indica* rice variety Kasalath consisting of about 65,000 clones with an average insert size of 106 kb from only a single ligation. This BAC library covers sixteen haploid genome equivalents with 99.9 % probability of completion of the continuous overlapping contig in the rice genome. The quality of this BAC library was ascertained by retrieving the BAC clones for seven rice RFLP markers. The construction of the high-density colony filters covering the full BAC clones of this Kasalath library is in progress.

Segregation distortion has been documented in a wide range of organisms (Lyttle 1991, Xu *et al.* 1997). It would arise from the elimination of specific gametes or zygotes that are controlled by a lethal or partially lethal factor. Furthermore, selective fertilization has been indicated as a possible cause of segregation distortion in higher plants. During the construction of the rice molecular linkage map, using an F₂ population from a cross between the *indica* variety Kasalath and *japonica* variety FL134, a strongly distorted segregation was detected on the middle of chromosome 3. Cheng *et al.* (1996) calculated the position of this lethal factor locus between the rice RFLP markers, XNpb23 and XNpb15, using the maximum likelihood method and the expectation conditional maximization algorithm. In the same chromosomal region, distorted segregation of morphological and molecular markers was also reported (Nakagahra 1972, Harushima *et al.* 1996, Xu *et al.* 1997). To date, we were able to isolate a total of eleven individual BAC clones from the Kasalath BAC library using XNpb23 and XNpb15 RFLP markers. The length of the interval between the two markers was estimated to be 1.7 centimorgans (Saito *et al.* 1991). We are going to fill the physical gap between the isolated BAC clones by chromosome walking.

The transformation efficiencies of BAC clones into *E. coli* were reported to be much higher than the transformation of YAC clones into yeast (Shizuya *et al.* 1992, Woo *et al.* 1994). In this experiment, we could rapidly construct a rice BAC library using first size selected 150–300 kb DNA. The transformation efficiency was as high as 1.4×10^7 clones per μ g of plasmid DNA. On the other hand, the average insert size from a ligation which used 300–500 kb DNA fragments from the first size selection was 139 kb, and up to 400 kb of insert DNA

was isolated. However, the transformation efficiency was decreased by about 100-fold (data not shown). This indicates that the insert size of BAC clones is negatively correlated with the frequency of transformation as previously reported (Wang *et al.* 1995). Though the average insert size of the BAC library is affected by the quality of the starting high molecular weight DNA, we realized some important points in constructing a BAC library with moderate insert size with a high transformation efficiency; such as 1) thoroughly eliminating the small DNA fragments during size selection, 2) removing the DNA fragments much higher than the desired average insert size, 3) properly concentrating the size-selected DNA fragments for BAC vector ligation reaction.

The development of the BAC library system should be greatly enforced to provide rapid isolation of agronomically important genes from individual varieties of the same species or related species. Agronomically important traits such as disease resistance are specifically restricted to individual varieties that adapt to the special and different environments. A recent report by Leister *et al.* (1998) indicated that resistance gene homologues from rice and barley were frequently mapped on a nonsyntenic region between the cereal species and also that the copy number of these sequences was diversified intraspecifically. The same observation was also reported by Kilian *et al.* (1997). To isolate such important genes, it is necessary to construct genomic libraries from each of the individual varieties of interest. Once the optimum conditions are set up, a BAC library could be constructed rapidly as indicated in this report. The combination of a high-density molecular map flanking the target gene and the rapid construction of the BAC library with moderate insert size for chromosome landing and walking should be the most promising procedure for map-based cloning.

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