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Construction of a bacterial artificial chromosome (BAC) library and identification of overlapping BAC clones with chromosome 4-specific RFLP markers in rice

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Abstract To facilitate construction of physical map of the rice genome, a bacterial artificial chromosome (BAC) library of IR64 genomic DNA was constructed. It consists of 18 432 clones and contains 3.28 rice genomic equivalents. The insert size ranged from 37 to 364 kb with an average of 107 kb. We used 31 RFLP markers on chromosome 4 to screen the library by colony hybridization. Sixty eight positive clones were identified with 2.2 positive clones per RFLP marker. The positive clones were analyzed to generate 29 contigs whose sizes ranged from 50 to 384 kb with an average of 145.6 kb. Chromosome walking was initiated for ten contigs linked to resistance genes. Thirty eight BAC clones were obtained and two contigs were integrated. Altogether, they covered 5.65 Mb (15.1%) of chromosome 4. These contigs may be used as landmarks for physical mapping of chromosome 4, and as starting points for chromosome walking towards the map-based cloning of disease resistance genes which were located nearby.

Key words BAC library · Landmark for physical mapping · Map-based gene cloning

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Introduction

Rice (*Oryza sativa* L.) is becoming a model crop among cereals for genome research because of its relatively small genome size (4.5×10^8) (Arumuganathan and Earle 1991), the availability of high-density DNA-marker maps (Causse et al. 1994 and Kurata et al. 1994), and the collinearity of its genes with those of other crops in the grass family (Kurata et al. 1994; Moore 1995). Thus, research on the rice genome will not only enhance our understanding of the genomic structure of rice but will also offer new tools for genome mapping, and gene cloning in other cereal crops (Gale et al. 1996).

An international effort is being made to study the rice genome. Rice genome projects have been initiated in many countries including Japan, China, Korea, and the European community. One of the central themes of these projects is the development of a highly reliable and high-resolution physical map of rice. Different cloning systems, including yeast artificial chromosome (YAC) (Burke et al. 1987) and bacterial artificial chromosome (BAC) (Shizuya et al 1992) techniques, have been used to prepare rice genomic libraries containing long segments of DNA (Umehara et al 1995; Wang et al 1995; Zhang et al 1996). Overlapping clones (contigs) can be generated via colony screening, chromosome walking, DNA fingerprinting, or sequence-tag-site (STS)-content mapping, using these libraries.

As part of the international efforts on rice genome research, we initiated the construction of a BAC library. We chose the BAC system because it has distinct advantages over the YAC system including: easy handling of the *Escherichia coli* host, high transformation efficiency, easy isolation of insert DNA from *E. coli* cells, a proper size (100–150 kb) for regular agarose-gel analysis and a low frequency of chimeric clones (Shizuya et al. 1992; Woo et al. 1994; Wang et al. 1995). Consequently, the BAC system may be more

efficient than a YAC system in generating a high-resolution physical map and identifying the minimum tilling path, i.e. the minimum number of overlapping BAC clones covering the maximum length of the rice genome.

To integrate the physical map with the genetic map, it is necessary to place some BAC clones onto a DNA marker map. These BAC clones then become landmarks of a physical map that marks the chromosomal numbers and positions. Landmarks can be generated either by STS-content mapping or colony hybridization with RFLP markers. If the STS and RFLP markers are linked to agriculturally important genes, the landmarks provide the initial points for chromosome walking and the eventual cloning of target genes.

In this report, we describe the construction and characterization of a rice BAC library based on DNA isolated from IR64, the most popular rice variety in the world. Furthermore, we identified overlapping BAC clones via colony hybridization with RFLP markers on chromosome 4 where several disease and insect resistance genes have already been mapped with RFLP markers (Mohan et al.) 1994; Wang et al. 1994; Yoshimura et al. 1995; Sebastian et al. 1996).

Materials and methods

Construction of a BAC library

Preparation of HMW and partially digested DNA

High-molecular-weight (HMW) DNA was extracted from rice tissue as described by Wang et al. (1995) with minor modifications. Nuclei were isolated from 200 g of 4-week-old leaves of IR64. The nuclear pellet was re-suspended in 2.5 ml of SCE buffer (1 M sorbitol, 0.1 M sodium citrate, 60 mM EDTA pH 7.0) and embedded in an equal volume of 2% low-melting-point (LMP) agarose. After proteinase-K treatment for 72 h at 50°C, the agarose plugs were dialyzed twice against TE buffer plus 1 mM of PMSF (phenylmethyl sulfonylfluoride) at 50°C for 1 h, and equilibrated with *Hin*dIII buffer twice at room temperature for 1 h. After removing the *Hin*dIII buffer, the plugs were melted at 65°C for 10 min and kept at 37°C for 5 min. Five units of *Hin*dIII per plug were added and incubated at 37°C for 1 h. The reaction was stopped by adding 1/10 vol of 0.5 M EDTA. Two or three plugs were loaded into a well of 1% LMP agarose gel and fractionated by pulse-field gel electrophoresis (PFGE), (CHEF DR III system BioRad, USA). First-size selection employed a switch-time ramping from 90 to 130 s, 4.5 V/cm with a 120° pulse angle at 14°C for 18 h. The gel containing DNA of 200–600 kb was cut placed on a 1% LMP agarose gel for second-size selection by switch-time ramping from 5 to 10 s, 4.5 V/cm with a 120° pulse angle at 14°C for 10 h. After PFGE, the condensed DNA band was cut from the gel and the gel slices were dialyzed twice against TEN buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl) for 2 h. The gel slices were then melted at 65°C for 10 min, and transferred to 40°C for 5 min. One unit of agarase (New England Biolabs, USA) per 100 µl was added and incubated at 40°C for 1.5 h. After a 1-h digestion, 2 µl of DNA solution was removed to check DNA concentration by running on a 0.6% agarose gel with a known concentration of undigested λ DNA.

Preparation and de-phosphorylation of vector DNA

BAC vector pBelBAC 11, in *E. coli* strain DH10B, was kindly provided by Dr. H. Shizuya, California Institute of Technology, USA. LB media (50 ml) containing 30 µg/mg of chloramphenicol was inoculated with a single colony of *E. coli* containing pBeloBAC 11 and incubated at 30°C overnight. Six liters of LB containing 30 µg/ml of chloramphenicol were inoculated with the overnight culture (30 ml) and incubated at 30°C for 35 h. The cells were harvested and vector DNA extracted by alkaline lysis as described by Sambrook et al (1989). Plasmid DNA was purified by cesium-chloride density gradient centrifugation in the presence of ethidium bromide in a Beckman VTi 65.2 rotor at 55 K rpm, at 20°C for 16 h. The plasmid band was removed from the density gradient and ethidium bromide was removed by several extractions with water-saturated isoamyl alcohol. The DNA sample was diluted 5-fold with TE and precipitated with ethanol. The pellet was washed and dissolved in TE buffer. The vector DNA was de-phosphorylated with HK phosphatase (Epicentre Technologies) as described in manufacturer's manual.

Ligation and transformation

The ligation was carried out in 100-µl vol in which the molar ratio of partially digested HMW DNA to de-posphorylated vector DNA was 1 : 5. Ligation was carried out with 1.5 units of ligase (Pharmacia, USA) at 16°C overnight. Before electroporation, the ligation mixture was dialyzed against TE in an Ultrafree-MC filter unit (Millipore, USA) at 4°C for 48 h until the volume had decreased to one-third of the original. One microliter of ligation mixture was used to transform DH10B, (GIBCOBRL, USA) by electroporation using a Gene Pulser (Bio-Rad, USA). Electroporation conditions were as follows: 1.25 kV field strength (0.2 cm diameter cuvette, 2.5 kV setting), 800 Ω resistance, 25 µF capacitance. After transformation, the cells were immediately transferred to 1 ml of SOC (2% bacto-tryptone, 0.5% bacto-yeast extracts, 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 for 1 h with shaking (225 rpm). The cells were spread on LB plates containing 12.5 µg/ml of chloramphenicol. For color selection, 10 µl of IPTG (20% w/v isopropylthio-β-D-galactoside) and 40 µl of X-gal (2% w/v 5-bromo-4-chloro-3-indolyl-β-galactoside) were spread on the plates. Plates were incubated at 37°C for 32–48 h. The recombinants (white colonies) were picked into 384-well microtiter plates containing 60 µl of LB freezing media [36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH4)_2$ SO_4, 4.4% (v/v) glycerol, 12.5 µg/ml chloramphenicol]. After incubating at 37°C for 32–38 h, the library was replicated twice and stored in three different −80°C freezers. Each BAC clone was designated by a clone address XYZ; X for plate number, Y for row number and Z for column number.

Screening of BAC library

Preparation of a high-density filter

Twelve of 10 × 12 cm Hybond filters (Amersham USA) were placed on three layers of Whatman filter saturated with LB. Each filter was spotted with BAC clones using a 384-well replica (V and P Scientific INC, USA) with four different microtiter plates so that a total of 1 536 BAC clones were inoculated onto a single filter. The filters were transferred onto trays containing LB agar with 20 µg/ml of chloramphenicol, and were incubated at 37°C for 12–18 h until the colonies were about 2 mm in diameter. Each filter was processed by placing it, colony side up, on two layers of 3-mm Whatman filters saturated with the following solutions: (1) 10% SDS, 5 min; (2) 0.5 M NaOH, 1.5 M NaCl, 5 min; (3) 0.5 M Tris-HCl (pH 7.4), 1.5

M NaCl, 1mM EDTA (pH 8.0), 5 min, twice; (4) 2 × SSC, 0.1%SDS, 5 min; (5) 2 × SSC, 5 min; (6) removal of the residual solution on dry Whatman filters, 1 min; and (7) 0.4 M NaOH, 20 min. Finally, the filters were washed twice by 1000 ml of 5 × SSC 0.2% SDS for 20 min each wash and 1000 ml of 2 × SSC twice for 5 min each time with gentle shaking.

Colony hybridization

Twelve filters were pre-hybridized in two boxes with 30 ml of phosphate hybridization solution [0.5 M Na_2PO_4 (pH 7.2), 1 mM EDTA, 7% SDS, 100 μg/ml denatured sheared salmon sperm DNA] at 65°C for 4–6 h. RFLP probes of chromosome 4 were kindly provided by Drs. S. D. Tanksley and S. McCouch, Cornell University, USA. The amplified products of RFLP markers were labeled by a hexamer random primer kit (Rediprimer, Amersham, USA) with ^{32}P-α-dCTP (Amersham, USA). The labeled probe was directly added to the pre-hybridization solution and hybridized at 65°C overnight. After hybridization, the filters were washed by 0.2 M phosphate buffer containing 0.1% SDS at 65°C twice for 20 min each time with gentle shaking. The filters were exposed to X-ray film (Kodak X-mat AR) for 8–24 h depending on the signal intensity.

Identification of overlapping clones and the assembly of contigs

The positive BAC clones were picked from the library and inoculated to 10 ml of LB containing 12.5 μg/ml of chloramphenicol and incubated at 37°C overnight. BAC DNA of individual clones was prepared by the standard alkaline-lysis method (Sambrook et al. 1989). Plasmid DNA was digested by *Hin*dIII for Southern blotting to identify overlapping clones and to assemble contigs. To check the size of each BAC clone, plasmid DNAs were digested by *Not*I and separated by PFGC in 0.5 × TAE buffer with the following setting: switch-time ramping from 3 to 8 s, voltage 9 V/cm with 100° pulse angle at 14°C for 3 h. DNA digested *Hin*dIII was separated on an 0.8% agarose gel and transferred onto Hybond filters. An RFLP marker and a BAC clone in each group of overlapping clones were chosen as probes to hybridize the filters in order to assemble the contigs.

Chromosome walking

The entire BAC clone DNA at both ends of contigs was used as a probe to screen the BAC library. The BAC DNA was digested by endonulecease before labeling. The positive clones were verified by hybridization and their integration into the contigs was based on the pattern of the DNA fingerprinting of positive BAC clones, as described above.

Results

Construction and characterization of a rice BAC library

The rice BAC library of IR64 consisted of 18 432 clones. The insert size distribution of 80 BAC clones, most of which were identified to be positive by hybridization with RFLP markers, was determined by digestion with *Not*I and separation by PFGE (Fig. 1). The largest and smallest inserts were 364 kb and 37 kb,

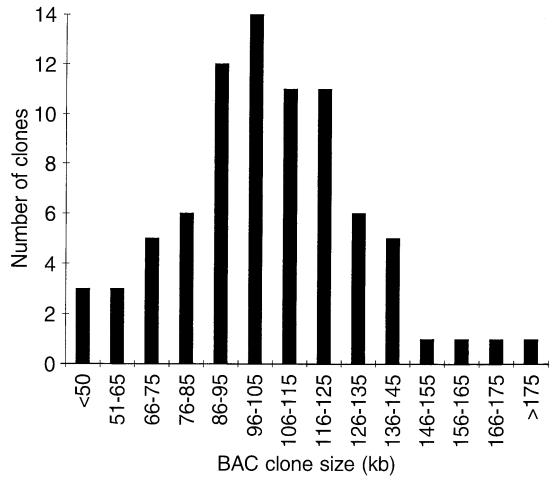


Fig. 1 Insert-size distribution of 80 BAC clones taken randomly from the BAC library

respectively. Out of 80 BAC clones, 75% of inserts distributed between 76 and 135 kb with an average of 107 kb. More than 650 BAC clones were checked and it was found that all of them contained inserts, indicating a very high percentage of clones in the library containing inserts. Thus, the library was estimated to have a 3.28 genome coverage based on a genome size of 4.5×10^8 bp (Arumuganathan and Earle 1991).

To characterize the insert of BAC clones, we randomly chose 15 rice BAC clones which we digested with *Not*I and separated by PFGE. The gel was stained with ethidium bromide (Fig. 2A). The DNA was blotted onto a nylon membrane and hybridized with total genomic DNA. The results indicated that most of the DNA bands can be hybridized with ^{32}P-dCTP-labeled rice genomic DNA at high-stringency washing condition (Fig.2B). Some of the bands showed a strong hybridization signal, which may be caused by a repetitive DNA sequence. Others showed a weak hybridization signal, which could be due to low- or single-copy DNA in the rice genome. The fact that each BAC clone contained different inserts, and different hybridization signals, implies that the rice BAC library may be representative and suitable for physical mapping and the map-based cloning.

The transformation conditions directly affected the transformation efficiency, insert size, and the frequency of BAC clones with no inserts (false positives). The most suitable transformation conditions which were used in this study are given in Materials and methods. Deviation from this could result in a lower transformation efficiency, increased false-positives or a smaller insert size (Table 1). For example, a pulse time longer than 25 ms could produce less colonies per transformation and a lower frequency of recombinants. On the other hand, When the pulse time was too short, e.g. less

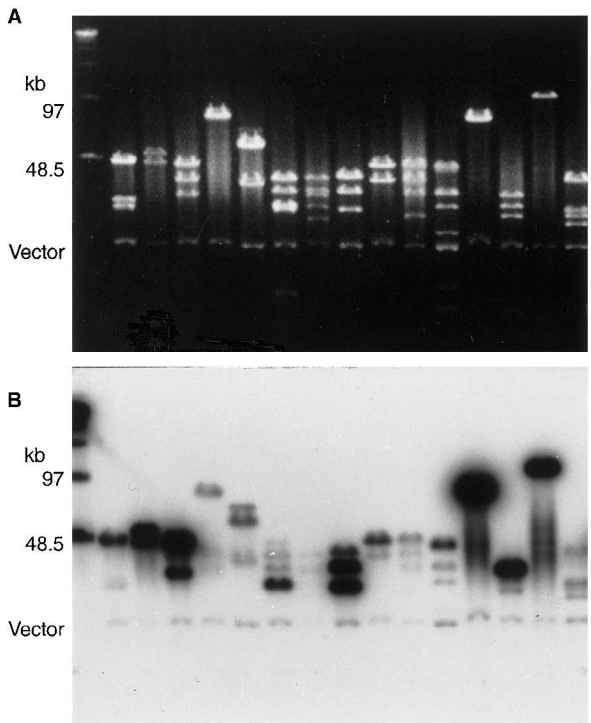


Fig. 2A, B Analysis of rice BAC clones by PFGE. **A** Ethidium bromide-stained agarose gel showing 15 BAC clones digested with *Not*I and separated by PFGE. **B** An autoradiograph of the same gel hybridized with total rice genomic DNA

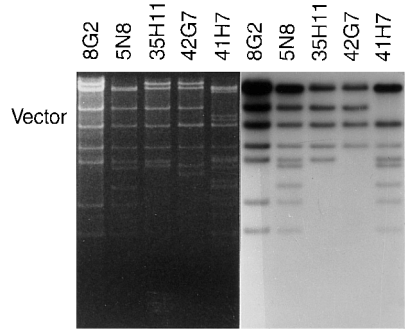


Fig. 3 Restriction and Southern analysis of positive BAC clones identified by RG449. DNA from five BAC clones, 8G2, 5N8, 35H11, 42G7 and 41H7, were digested with *Hin*dIII, separated on an 0.8% agarose gel, transferred to a nylon membrane, and probed with a labeled BAC clone, 5N8. The left panel shows the restriction fragment pattern of clones digested by *Hin*dIII stained with ethidium bromide, and the right panel shows a Southern analysis of the same gel

than 10 ms, both the insert size and the transformation efficiency were much less than optimal (Table 1). We have also tested the effect of voltage. High voltage did not increase insert size and transformation efficiency but did reduce the number of recombinants (data not shown). Initially, we used an *E. coli* pulser (Bio-Rad, USA) for transformation. As pulse time cannot be adjusted with an *E. coli* pulser, our test indicated that the *E. coli* pulser is not suitable for transformation of BACs with large inserts.

Screening of the rice BAC library with RFLP markers

Five sets of high-density filters, each consisting of 12 filters, were prepared and used for library screening. Using 31 chromosome-4-specific RFLP markers to screen the library, 68 positive BAC clones were identified. Positive clones were verified by restriction digestion and Southern analysis of BAC DNA (Fig. 3). At least one BAC clone was identified for each probe. In some cases, up to six clones were identified per RFLP marker probe. Overall, an average of 2.2 BAC clones per marker was identified. The insert size of all positive clones was determined by *Not*I digestion and PFGE. The insert sizes and numbers of *Not*I sites of all positive BAC clones are summarized in Table 2. The number of *Not*I sites ranged from 0 and 7 with an average of 1.34 sites.

BAC contigs linked to resistance genes on chromosome 4

Many disease- and insect-resistance genes have been mapped on chromosome 4 (Mohan et al 1994; Wang

Table 1 The effects of pulse time on the transformation efficiency and insert size of BAC clones

Pulse time[a] (m s)	Voltage[b] (kV)	Colony[c]	Recombinants (%)	Insert size (kb)	False-positives[d] (%)
5	1.25	45	97.8	93	0
10	1.25	125	87.2	109	0
15	1.25	137	86.9	118	0
20	1.25	129	89.9	104	0
25	1.25	59	67.8	95	2.5

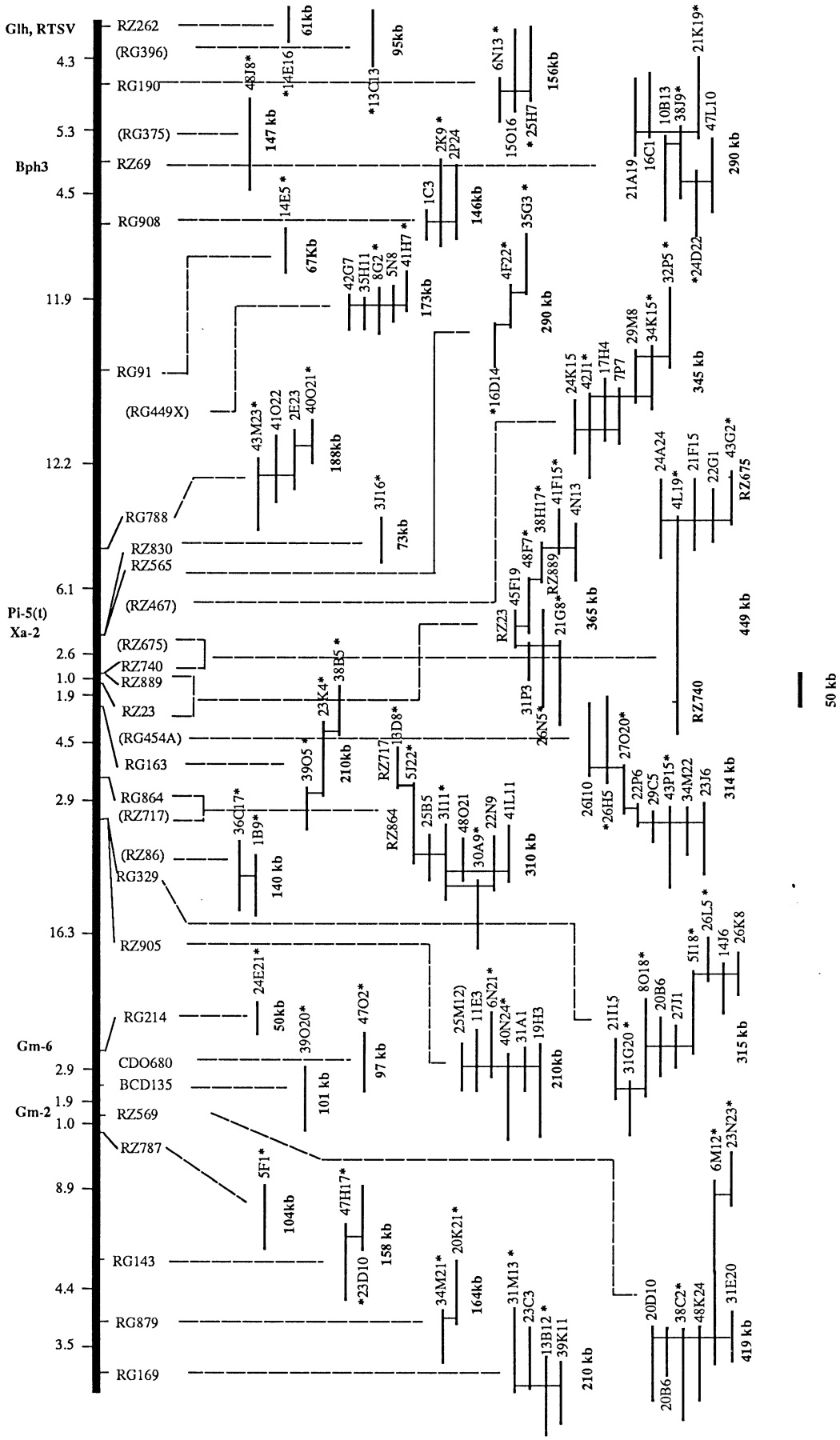
[a] Pulse time means resistance × capacitance
[b] Field strengths, kV/cm
[c] Number of colonies per transformation using 1 μl of the same ligation
[d] False-positives, BAC clones of white color, but without an insert

Table 2 BAC clones identified by RFLP markers and chromosomes walking of chromosome 4

RFLP marker	B4C clones[a]	Insert size (kb)	*Not*I sites
RZ905	6N21	105	0
	40N24	146	2
	*25M12	74	1
	48J9	156	2
	31A1	70	1
	11E3	95	1
RG329	5I18	128	2
	8O18	141	3
	*20B6	89	4
	27J1	67	2
	26L5	67	0
	14J6	77	1
	26K8	70	0
	31G20	101	1
	21I15	93	0
RG214	24E21	50	0
RZ69	24D22	102	1
	38J9	119	1
	*47L10	128	1
	21A19	130	2
	16C1	104	1
	10B13	142	2
	21K19	90	0
RZ467	17H4	97	0
	*29M8	91	1
	7P7	90	2
	24K15	73	0
	42J1	140	2
	34K15	105	1
	32P5	131	1
RG190	25H7	120	2
	15O16	129	1
	*6N13	70	1
RG454	*22P6	37	0
	27O22	70	1
	26I10	115	2
	26H5	136	1
	29C5	42	0
	43P15	132	2
	34M22	76	2
	23J6	117	2
RZ169	39K11	100	2
	*13B12	124	2
	*23C3	105	3
	31M13	138	2
RG788	43M23	118	2
	41O22	113	1
	*2E23	94	1
	40O21	87	1
RZ569	6M12	304	5
	20D10	116	1
	*19H3	79	0
	31E20	86	2
	38C2	136	1
	48K24	121	1
	23N23	85	0
RG449	8G2	142	0
	*5N8	111	1
	35H11	104	1
	42G7	115	1
	41H7	133	1
RZ787	5F1	104	2
RZ717	13D8	61	1
RG864	25B5	61	0
	3I11	116	2
	15J22	105	1
	48O21	69	0
	30A9	110	1
	22N9	89	1
	41L11	94	1
RZ262	14E16	61	0
RZ23	45F19	60	0
	48F7	86	0
	31P3	61	0
	26N5	160	2
	21G8	140	2
RZ889	38H17	61	0
	41F15	73	0
	4N13	90	1
RZ740	4L19	364	7
	43G2		
	22G1	86	1
	21F15	67	1
	24A24	124	2
RZ675	43G2	87	0
RZ565	4F22	71	1
	16D14	124	2
	35G3	102	5
RZ879	34M21	79	0
	*20K21	101	2
RZ830	3J16	73	1
RG143	*23D10	104	2
	47H7	120	3
RG163	23K4	123	3
	39O5	70	0
	38B5	70	0
RG91	14E5	67	0
RG396	13C13	95	0
RG375	48J8	147	3
RG908	1C3	48	0
	*2K9	146	1
	2P24	121	1
RZ86	*36C17	113	6
	1B9	100	5
CDO680	4702	97	0
BCD135	*39O20	101	1

[a] BAC clones with a star (*) was used as probe. BAC clones in bold were obtained by chromosome walking

et al. 1994; Yoshimura et al. 1995; Sebastian et al. 1996). To identify BAC clones closely linked to these genes, chromosome walking was initiated from ten contigs (Table 2 and Fig. 4). A total of 38 BAC clones were identified to overlap with original contigs by DNA fingerprinting of BAC clone DNA. Two landmarks clones, RZ740 and RZ675, were integrated to a contig with a physical distance of 449 kb (Fig. 4). These BAC clones are linked to the blast resistance gene, *Pi-5*(t) (Wang et al. 1994), and the bacterial blight resistance gene, *Xa-2* (Yoshimura et al. 1995). BAC clones identified by RZ569 and RG214 are linked to two gall midge resistance genes, *Gm2* and *Gm6* (Mohan et al. 1994;


Glh, RTSV
Bph3
Pi-5(t)
Xa-2
Gm-6
Gm-2
RZ262
(RG396)
RG190
(RG375)
RZ69
RG908
RG91
(RG449X)
RG788
RZ830
RZ565
(RZ467)
(RZ675)
RZ740
RZ889
RZ23
(RG454A)
RG163
RG864
(RZ717)
(RZ86)
RG329
RZ905
RG214
CDO680
BCD135
RZ569
RZ787
RG143
RG879
RG169
4.3
5.3
4.5
11.9
12.2
6.1
2.6
1.0
1.9
4.5
2.9
16.3
2.9
1.9
1.0
8.9
4.4
3.5
61kb
95kb
147 kb
146kb
67Kb
173kb
290 kb
345 kb
188kb
73kb
365 kb
449 kb
210kb
314 kb
140 kb
310 kb
50kb
97 kb
101 kb
210kb
315 kb
104kb
158 kb
164kb
210 kb
419 kb
156kb
290 kb
50 kb

J. Bennett, personal communication). RZ262 identified a BAC clone, 14E16, which was linked to genes for tungro and green leafhopper resistance. Seven BAC clones, identified by RZ69 and chromosome walking, formed a 290-kb contig. This contig is closely linked to the brown planthopper resistance gene, *Bph3* (N. Huang, unpublished data).

RFLP landmark for a physical map of rice chromosome 4

Based on Southern-blot analysis and restriction fingerprinting, the 102 positive clones identified by 31 RFLP markers on chromosome 4 and chromosome walking formed 28 contigs (Fig. 4), which could serve as landmarks for future physical map construction and the isolation of resistance genes. Two RFLP markers, RZ23 and RZ889, which were 1 cM apart (Causse et al. 1994), were physically linked together through a BAC clone, 48F7, to form a contig of three BAC clones with a physical distance of 175 kb (Fig. 4). This contig was extended to 365 kb by chromosome walking from both directions. Four BAC clones identified by two other RFLP markers, RZ864 and RZ717, were also connected together to form a contig covering 230 kb which was extended to 310 kb by chromosome walking. Two RFLP markers, RZ675 and RZ740, were physically linked in a 449-kb contig (Fig. 4).

Each of the 28 contigs consisted of 1–9 BAC clones. The largest and smallest contigs covered 449 kb and 50 kb, respectively, with an average of 201.7 kb. Altogether, the contigs covered 5.65 Mb of chromosome 4. Because some clones were redundant, 5.65 Mb of chromosome 4 could be represented by only 54 BAC clones (Fig. 4). Based on the chromosomal position of RFLP markers (Causse et al. 1994), the BAC contigs were integrated with the RFLP map (Fig. 4).

Discussion

The major advantages of the BAC system over other systems are its high transformation efficiency, the easy manipulation of insert DNA fragments, and the absence of chimerics (Shizuya et al. 1992; Wang et al. 1995). We constructed a library containing 18 432 clones using only two ligation mixtures of 100 μl each. The maximum number of BAC clones per transformation (1-μl ligation) can be more than 700 BAC clones. At the same time, the largest insert identified is about 364 kb. These results agreed with those of others (Shizuya e al. 1992; Wang et al. 1995) in that the BAC system is capable of rapidly producing a large-insert library, and provides for the faithful propagation of large insert fragments up to 350 kb. In addition, we successfully screened the BAC library with RFLP markers and the assembled contigs, confirming that the BAC system will be a very useful tool to generate a high-resolution physical map as shown by Schmitt et al. (1996).

Several lines of evidence indicated that the present BAC library is representative and is suitable for the high-resolution physical mapping of the rice genome:

(1) From the present library, DNA from several hundred BAC clones has been isolated. The insert size is from 37 to 364 kb. We have not yet found any clone that does not carry an insert.
(2) Screening of the BAC library with 31 RFLP markers identified 68 positive clones, yielding an average of 2.2 clones per probe. All RFLP markers used picked up at least one BAC clone.
(3) Some closely linked RFLP markers were physically connected through contigs of overlapping BAC clones.
(4) Southern-blot analysis of BAC clones showed that different BAC clones carried inserts of different size and origin (Fig. 2).

Using the library, small contigs were generated for BAC clones identified by RFLP markers on chromosome 4 and extended by chromosome walking. These would be useful as starting points in constructing a physical may for the entire chromosome. Furthermore, several genes for gall midge (Mohan et al. 1994), green leaf hopper (Sebastian et al 1996; N. Huang unpublished data), tungro (Sebastian et al. 1996), brown planthopper (N. Huang, unpublished data), blast (Wang et al. 1994), and bacterial blight resistance (Yoshimura et al 1995) have been mapped on chromosome 4 with the RFLP markers used here. Therefore the BAC landmarks can be used to initiate chromosome walks toward these genes. The contigs may also serve as anchors to place overlapping BAC clones onto the chromosome. The overlapping clones can be identified by DNA clone-analysis techniques.

The BAC system is capable of quickly generating a physical map of high quality and resolution. Assuming the size of rice chromosome 4 is 3.75×10^4 kb (the average size of all chromosomes) the contigs generated from BAC clones identified with the 28 RFLP markers provided a coverage of 15.1% of chromosome 4 (5.65×10^3 kb). Furthermore, the contigs were verified by Southern hybridization and therefore should be highly reliable. As the DNA could easily be isolated

Fig. 4 The partial and genetic map of chromosome 4. The location and map distances of RFLP markers are based on Causse et al. (1994). The map distances are presented in cM to the left of the chromosome. The 28 contigs of chromosome 4 are shown to the right of the chromosome. The BAC clones forming non-redundant overlaps are indicated by a *star* (*)

from BAC clones, all clones picked by colony hybridization were examined by DNA fingerprinting with *Hin*dIII digestion. The DNA fingerprints showed the overlap and non-overlap portion of the BACs (Figs. 3 and 4) thus providing a high-resolution physical map from which the minimum tilling path can be easily identified (Fig. 4).

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