Molecular Breeding **1**: 79–89, 1995.

Construction and characterization of a rice YAC library for physical mapping

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Received 1 March 1994; accepted in revised form 20 July 1994

Key words: physical mapping, rice, YAC

Abstract

Genomic libraries of rice, *Oryza sativa* L. cv. Nipponbare, in yeast artificial chromosomes were prepared for construction of a rice physical map. High-molecular-weight genomic DNA was extracted from cultured suspension cells embedded in agarose plugs. After size fractionation of the *Eco* RI- and *Not* I-digested DNA fragments, they were ligated with pYAC4 and pYAC55, respectively, and used to transform *Saccharomyces cerevisiae* AB1380. A total of 6932 clones were obtained containing on average ca. 350 kb DNA. The YAC library was estimated to contain six haploid genome equivalents. The YACs were examined for their chimerism by mapping both ends on an RFLP linkage map. Most YACs with *Eco* RI fragments below 400 kb were intact colinear clones. About 40% of clones were chimeric. Genetic mapping of end clones from large size YACs revealed that the physical distance corresponding to 1 cM genetic distance varies from 120 to 1000 kb, depending on the chromosome region. To select and order YAC clones for making contig maps, high-density colony hybridization using ECL was applied. With several probes, at least one and at most ten YAC clones could be selected in this library. The library size and clone insert size indicate that this YAC library is suitable for physical map construction and map-based cloning.

Introduction

In order to understand the genome structure of organisms and to isolate important genes, physical maps are essential, particularly for genomic reconstitution with DNAs and for linking genetic maps of phenotypic traits with DNA fragments. Yeast artificial chromosomes (YAC) [4] are effective tools for the preparation of ordered genomic clone libraries because they can clone large DNA fragments of several hundred kb. YACs have already proved to be very useful in physical mapping and gene isolation in many genome research programs. In human chromosome mapping, ordered YAC libraries covering the long arm of chromosome 21 [7] and chromosome Y [14] were constructed using STS markers. The ordering of YACs to cover whole human genome by fingerprinting and other methods is also proceeding [5, 8]. Several human genes and specific chro-

mosomal regions, for example the familial adenomatous polyposis locus [24], have been isolated and characterized using YACs. Ordered genomic libraries of *C. elegans* were constructed by linking cosmid contigs with YACs [9]. In plant genome research, construction and utilization of YAC libraries were reported on *Arabidopsis thaliana* [19, 17, 35], *Daucus carota* (carrot) [19], *Lycopersicon esculentum* (tomato) [26], *Zea mays* (maize) [11] and *Hordeum vulgare* (barley) [10], etc. In *Arabidopsis*, YAC libraries have been utilized for physical mapping [31] and for gene isolation by a map-based cloning method [1]. The tomato YAC library has also helped to isolate important phenotypically identified genes, including disease resistance genes [25, 26].

Rice is one of the most important crops in the world, and has been considered as a model organism for cereal plants genome research for several reasons. First, rice has a relatively small-size genome (haploid 4×10^8 bp) [2]. Second, both genetic maps with many phenotypical traits [23] and marker-condensed RFLP linkage maps [29, 32, 34] are well developed in rice. Third, transformation and regeneration systems are established for several varieties. Fourth, comparison of rice genome with other cereals is likely to yield valuable information on gene synteny [27]. Finally, physical maps of several rice linkage groups have been created using an *in situ* hybridization method [18].

In this paper we report the first successful construction of a rice YAC library with an average size longer than 300 kb. Several YAC end clones were mapped in the RFLP linkage map and comparisons of physical and genetic distances could be done. Qualitative analysis of the library (YAC size, chimeric clone frequency and genome coverage) showed that this YAC library is promising for physical mapping and gene isolation.

Materials and methods

Plant cell culture

Suspension cultured cells from germinating rice seeds (*Oryza sativa* L. cv. Nipponbare) [30] were used for isolating protoplasts. Seeds of Nipponbare were dehulled, sterilized with ethanol and sodium hypochlorite and placed on MS medium [28] containing 2 μg/ml 2,4-D, Fe-EDTA diluted to 1/5 of basal MS, 3% sucrose and 0.3% Gelrite. After incubation at 25 °C for about one month, induced calli were transferred to N6 medium [6] supplemented with Mo, Co, Cu and vitamins at the same concentrations as in the MS medium. Cultured cells were grown with constant agitation of 100 rpm at 25 °C in N6 medium which was changed every seven days. For obtaining actively growing cells capable of regeneration, the suspended calli were strained through stainless steel mesh (20 mesh) with a spatula every two weeks to make small particles.

Preparation of rice high-molecular-weight DNA

Rice protoplasts were isolated as described previously [30]. One week after straining the cells through stainless steel mesh, cells were transferred to MS medium containing 6% sucrose and cultured for 5 days at 25 °C. Then the cells were rinsed with a washing solution (0.5 M mannitol, 0.1 mM $CaCl_2$) and treated with 2% Cellulase 'Onozuka' RS (Yakult Co.) and 0.05% Pectolyase Y-23 (Seishin Pharmaceutical Co.) in the washing solution at 30 °C for 3 h. The formed protoplasts were filtered through 30 μm nylon mesh, rinsed twice with washing solution, and resuspended in the washing solution at a final concentration of 1.2×10^8 cells/ml. Rice high-molecular-weight DNA was extracted according to the agarose plug method [20]. The suspended protoplasts were mixed with an equal volume of 1% low-melting-point agarose (ICI), poured into molds (Pharmacia), and solidified at −20 °C for 7 min. Agarose plugs were washed twice with ES solution (1% *N*-lauryl sarcosine, 0.5 M EDTA pH 9.5) for 30 min and treated with ES solution containing 0.5 mg/ml proteinase K at 50 °C for 48 h. The plugs were soaked in 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride for 1 h, washed twice in 10 mM Tris-HCl, 5 mM EDTA pH 7.5 for 2 h and then

stored at 4 °C in 10 mM Tris-HCl, 5 mM EDTA pH 7.5.

Digestion of rice high-molecular-weight DNA

Rice high-molecular-weight DNA was digested with *Not* I or partially digested with *Eco* RI under competition with *Eco* RI methylase [13]. The plugs were washed three times with 10 mM Tris-HCl pH 7.5 for 30 min, equilibrated twice with enzyme reaction buffer for 30 min at 4 °C (reaction buffer for *Not* I digestion: 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 0.01% Triton X-100, 0.01% bovine serum albumin, 0.5 mM spermidine; for *Eco* RI digestion: 100 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 80 μM *S*-adenosyl methionine, 0.5 μg/ml bovine serum albumin, 2.6 mM spermidine, 1 mM dithiothreitol). *Not* I digestion was carried out at 37 °C for 4 h with 1 unit of *Not* I added to one half of each plug containing ca. 3 μg of rice DNA. *Eco* RI digestion was carried out with 5 units of *Eco* RI and 200 units of *Eco* RI methylase added to one half of each plug at 37 °C for 4 h. Digested plugs were treated with proteinase K in ES solution and washed with 10 mM Tris-HCl pH 7.5, 10 mM EDTA.

Construction of YAC library

Digested rice DNA was size-fractionated with CHEF (contour-clamped homogeneous electrophoresis, BioRad) in 0.8% low-melting-point agarose Sea Plaque for 20 h at 14 °C, 3 V/cm, by switching every 20 s, in 0.5 × TBE (1 × TBE = 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA pH 8.3). Focusing bands were excised, washed with 10 mM Tris-HCl pH 7.5, 10 mM EDTA and with 10 mM Tris-HCl pH 7.5, 25 mM NaCl. pYAC vectors were digested with *Bam* HI and *Not* I (for pYAC55) or *Eco* RI (for pYAC4), and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer). The gel slices containing size-fractionated DNA were mixed with equal quantities of pYAC vector (54 μg for *Eco* RI digestion and 27 μg for *Not* I digestion) and melted at 65 °C for 10 min. The melted agarose solution was mixed with 1/4 volume of ligation solution (final concentration: 66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP pH 7.5, 2 units/μg DNA of T4 DNA ligase). Ligation reaction was done at 37 °C for 6 h and continued at 16 °C overnight. Ligated DNA was size-fractionated with CHEF at the same conditions, except that 1% low-melting-point agarose was used. The focused bands were excised out, washed with 10 mM Tris-HCl pH 7.5, 10 mM EDTA and with 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 25 mM NaCl. The gel slices were melted and treated with β-agarase (New England Biolab) at 40 °C for 3 h. These rice DNAs ligated with pYAC vectors transformed *Saccharomyces cerevisiae* AB1380 (Matα, *ura* 3, *trp* 1, *ade* 2-1, *can* 1-100, *lys* 2-1, *his* 5) with the spheroplast method [21, 3], modified by adding 10 μg of yeast transfer RNA (Sigma) as a carrier to 100 μl of spheroplasts. The transformants were stocked in 96-well microtiter plates at −80 °C as described by Imai and Olsen [21].

Examination of YAC lengths

High-molecular-weight DNAs of yeast transformants were prepared as described by Imai *et al.* [20]. These DNAs were electrophoresed with CHEF for 16 h 20 min, at 6 V/cm, by sequential switching from 9.8 to 114.75 s in 0.5 × TBE at 14 °C. The DNAs were blotted to Hybond N (Amersham) membranes by alkaline transfer method and linked to the membranes by UV irradiation. The length of YACs was examined by Southern blot hybridization probing with pYAC4 left-arm-specific sequence [20] labelled with digoxigenin-dUTP. Hybridization, washing and detection were carried out with a digoxigenin system according to the manufacturer's protocols (Boehringer).

Isolation of YAC end clones and RFLP mapping

YAC end fragments were obtained by the adapter-mediated polymerase chain reaction

(PCR) method [22]. DNAs were extracted from YAC clones cultured two days in AHC medium as described [21] and digested with endonucleases: *Pst* I, *Hin*d III, *Bfa* I, *Taq* I, *Dra* I, *Hin*c II, *Bst* UI, *Eco* RV, *Bam* HI or *Bgl* II. The digested DNA was ligated with dephosphorylated adapters. Six kinds of dephosphorylated adapters with different ends were used. The ends of the six adaptors were cohesive to *Pst* I digests, *Hin*d III digests (Takara Shuzo Co) *Taq* I digests, *Sau* 3 AI digests, *Bfa* I digests and blunt-end digests respectively. End-fragment amplification by PCR was carried out first with C1 primer on adapters (5′-GTACATATTGTCGTTAGAACGCG-3′) [22] and YAC left-arm primer-1 (5′-CGCGATCATGGCGACCACAC-3′) or YAC right-arm primer-1 (5′-ATATAGGCGCCAGCAACC-3′) [12]. Then the products were amplified further with C2 primer on adapters (5′-TAATACGACTCACTATAGGGAGA-3′) [22] and YAC left-arm primer-2 (5′-AAGTTGGTTTAAGGCGCAAGAC-3′) or YAC right-arm primer-2 (5′-CGCCCGATCTCAAGATTACG-3′) [12] located inside between C1 primer and YAC vector primer-1. The amplified fragments were ligated with pBluescript SK+. The cloned YAC end fragments were amplified by PCR, labelled by peroxidase and hybridized with rice genomic DNA (cv. Nipponbare, cv. Kasalath and F_2 progeny derived from a cross between Nipponbare and Kasalath). Hybridization and detection were done using the ECL system (Amersham) first to detect restriction fragment length polymorphism (RFLP) between parent DNAs. Then the YAC end fragments showing RFLP were analyzed for segregation of RFLP with 186 F_2 plants and their loci were determined on the RFLP map constructed earlier [29].

YAC screening with colony hybridization

A total of about 7000 YAC clones were dotted and cultured on membrane filters for colony hybridization. A total of 1636 YAC clones were transferred onto each Hybond N filter (12 cm × 8 cm, Amersham) with Biomeck 1000 (Beckman). The clones on filters were cultured on AHC plates containing 50 μg/ml of ampicilline for 3 days at 30 °C, equilibrated with 20% glycerol and stored at −80 °C as main filters. Replica YAC filters for colony hybridization were prepared by pressing the main filter and cultured at 30 °C overnight on AHC plates (according to Dr Yuji Kohara, personal communication). These filters were treated with Zymolyase 100T (Seikagaku Kogyo) in 1 M sorbitol, 100 mM sodium citrate pH 5.8, 50 mM EDTA, 15 mM DTT for overnight at 37 °C, denatured in 10% SDS for 5 min and in 0.5 M NaOH for 10 min, neutralized in 200 mM Tris-HCl pH 7.5, 25 mM NaCl for 5 min twice, soaked in 2× SSC, 25 mM NaCl for 5 min, and UV-linked. Colony hybridization with RFLP probes and detection was done using ECL system (Amersham). Candidate YAC DNAs from positive colonies were digested with endonucleases, electrophoresed and hybridized with RFLP markers to confirm whether those YACs were derived from the marker loci or not.

Results

Construction of YAC library

Rice high-molecular-weight DNA was extracted from protoplasts embedded in low-melting-point agarose plugs. The protoplasts used in this study were prepared from cultured suspension cells maintained for 4 months and were proven to have a high ability of regeneration. About 6×10^6 protoplasts embedded in a 100 μl of 0.5% agarose plug could produce ca. 6 μg DNA. Two kinds of endonucleases, *Eco* RI and *Not* I, were used for digestion of high-molecular-weight DNA to construct YAC libraries. We used 24 plug halves (about 72 μg of rice DNA) for *Eco* RI digestion and 12 plug halves (about 36 μg of DNA) for *Not* I digestion. A total of 5067 transformed clones containing *Eco* RI digests and 1865 clones carrying *Not* I digests were obtained and stored individually in wells of 96-well microtiter plates.

To estimate the size of YACs, high-molecular-weight DNAs were extracted from 167 clones,

electrophoresed with CHEF and blot-analyzed with pYAC4 left-arm-specific sequence as a probe. As shown in Fig. 1, the length of YACs on electrophoresis gels varied from 100 kb to more than 1 Mb. The size distribution of these fragments is shown in Fig. 2. The average size of the YACs was about 350 kb. Thus the *Not* I and *Eco* RI libraries can be estimated to cover about 2.4×10^9 bp which is six times the size of rice haploid genome (400 Mb).

It has been reported earlier that the cloning of large size DNAs in YACs is frequently accompanied by problems of deletion, rearrangement and chimerism [16]. Chimerism of YACs is the most serious problem for chromosome walking. To estimate the frequency of chimeric YACs in the library, both ends of the YACs were subcloned and mapped independently by RFLP linkage analysis [29]. We isolated 81 end clones from 45 YACs with *Eco* RI digests and 16 end clones from 8 YACs with *Not* I digests. Genomic Southern hybridization showed that only 31 end clones out of 97 showed RFLP. In 66 unmapped end fragments, 12 fragments showed monomorphic bands, 21 fragments showed multiple bands and 33 fragments showed too weak bands to be mapped. In the case of *Eco* RI-YACs, 6 pairs of YAC-end clones were located within 2.7 cM, but

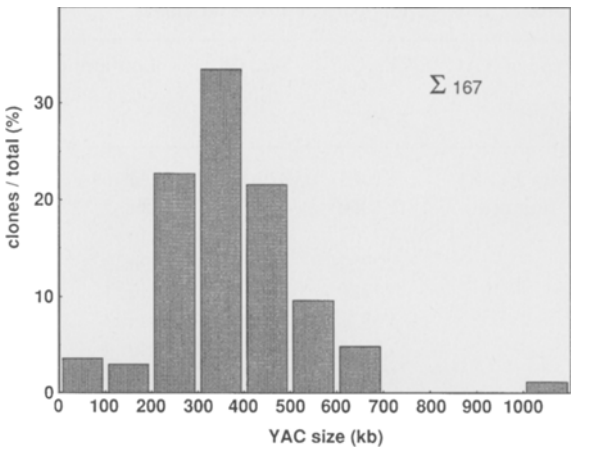

Fig. 2. Size distribution of 167 YACs in a rice YAC library. The X axis represents the size of YACs, the Y axis represents the percentage of clones with certain YAC size. Σ represents the number of clones examined. The average YAC size was about 350 kb, and the maximum size more than 1 Mb.

4 pairs of YAC ends were mapped far apart (Table 1). From these results, it was estimated that about 40% of the YACs in *Eco* RI library were chimeric. Most of the chimeric YACs were longer than 500 kb, but YACs shorter than 400 kb were colinear. The physical distance per cM varied between 120 kb and 1 Mb as estimated in Table 1 and Fig. 3. The distance per cM was

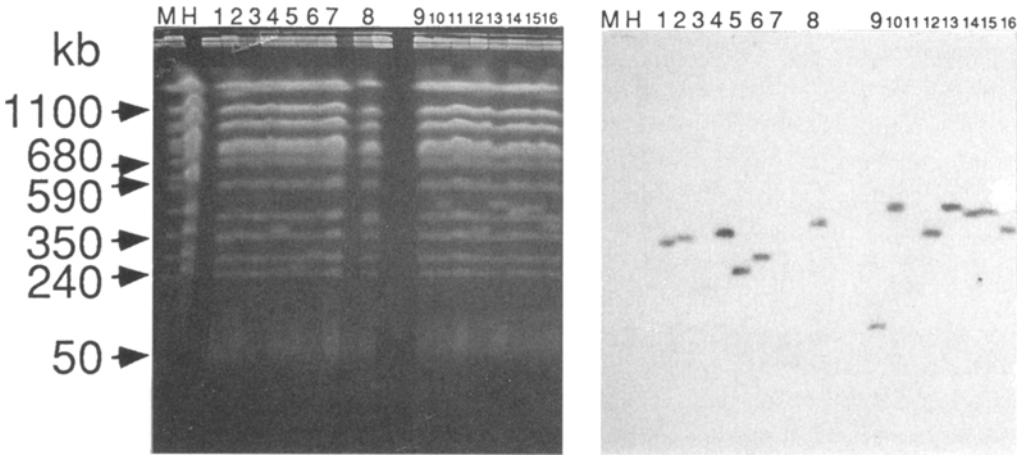

Fig. 1. Detection of YAC clones with large-size rice DNA. The panels show 16 YAC clones carrying fragments partially digested with *Eco* RI (lanes 1–16). Lanes: M, *S. cerevisiae* YNN295; H, *S. cerevisiae* AB1380 without YACs. Left panel: yeast chromosomes separated by pulsed-field gel electrophoresis. Right panel: hybridization images of the same YAC clones as in the left panel. YAC clones were detected by hybridization to pBR322 sequence of the YAC vector.

Table 1. Linkage mapping of YAC end clones.

Type of YAC	YAC	Size (kb)	Left-arm end clone mapped	Right-arm end clone mapped	Genetic distance (cM) between right and left ends	Ratio of physical distance to genetic distance (kb/cM)
With *Eco* RI fragment	Y1053	320	chr. 10	chr. 10	2.7	118
	Y1060	490	chr. 5	chr. 5	0.8	613
	Y2707	500	chr. 7	chr. 7	0.5	1000
	Y2757	440	chr. 2	chr. 2	0.8	550
	Y2820	300	chr. 1	chr. 1	2.1	143
	Y3870	300	chr. 3	chr. 3	2.4	125
	Y1065	600	chr. 4	chr. 3	chimeric	–
	Y2668	600	chr. 6 and 11	N.D.	chimeric	–
	Y2686	590	chr. 6	chr. 6	40 (chimeric)	–
	Y3635	420	chr. 6	chr. 4	chimeric	–
With *Not* I fragment	Y6854	600	chr. 11	chr. 12	chimeric	–
	Y6855	320	chr. 9	chr. 1 and 11	chimeric	–

about 100 kb in three YACs, longer than 500 kb in another three YACs and average was 425 kb. The longest distance was observed in the case of Y2707 having 1000 kb/cM distance located in the marker condensed region of chromosome 7. In the case of *Not* I digests, only two YACs were examined and both were chimeric. The *Eco* RI library contained long size YACs with an average length of 350 kb, over 60% of which were colinear clones. This library is thus suitable for physical mapping and chromosome walking.

Isolation of YAC clones by colony hybridization

As a result of screening by colony hybridization and confirmation by Southern blot hybridization, 40 YAC clones containing sequences of RFLP markers were isolated (Table 2). An example is shown in Fig. 4B, selected by probe C226A mapped on the chromosome 6. The bands A and B of lane N were mapped by RFLP mapping on C226A locus of chromosome 6 and on C226B locus of chromosome 7, respectively [29]. The band A appearing in Y3230 (lane 2) and Y4404 (lane 3) clones revealed that these YACs contain C226A sequence on chromosome 6. Another band of B found in lane N also appearing in Y2915 (lane 5) showed that this YAC clone carries C226B sequence on chromosome 7. In some cases false-positive clones, such as Y3370 and YN955 in lanes 1 and 4, respectively, were confirmed by Southern hybridization by showing no hybridization signals with C226 probe.

YAC clones possessing RFLP marker sequences of G124B, G264, G267, G30, G122, G329 and G44 located in chromosomes 4, 6 and 11 [30, 32] could also be isolated (Table 2). Thus, using colony hybridization with high density colony filters, one to eleven YAC clones carrying the same sequence as the RFLP marker could be isolated from the library constructed here. The number of selected YAC clones corresponded well to the frequencies expected in this library. These results show that our YAC library is of high quality, with enough clones screenable with various RFLP probes by colony hybridization.

Discussion

In order to clarify the rice genome structure, our rice genome research program has been constructing physical maps and linkage maps and performing large-scale cDNA analysis. Because rice is not only an important crop in itself, but also a useful model plant of cereals, understanding of rice genome structure will be beneficial in genome

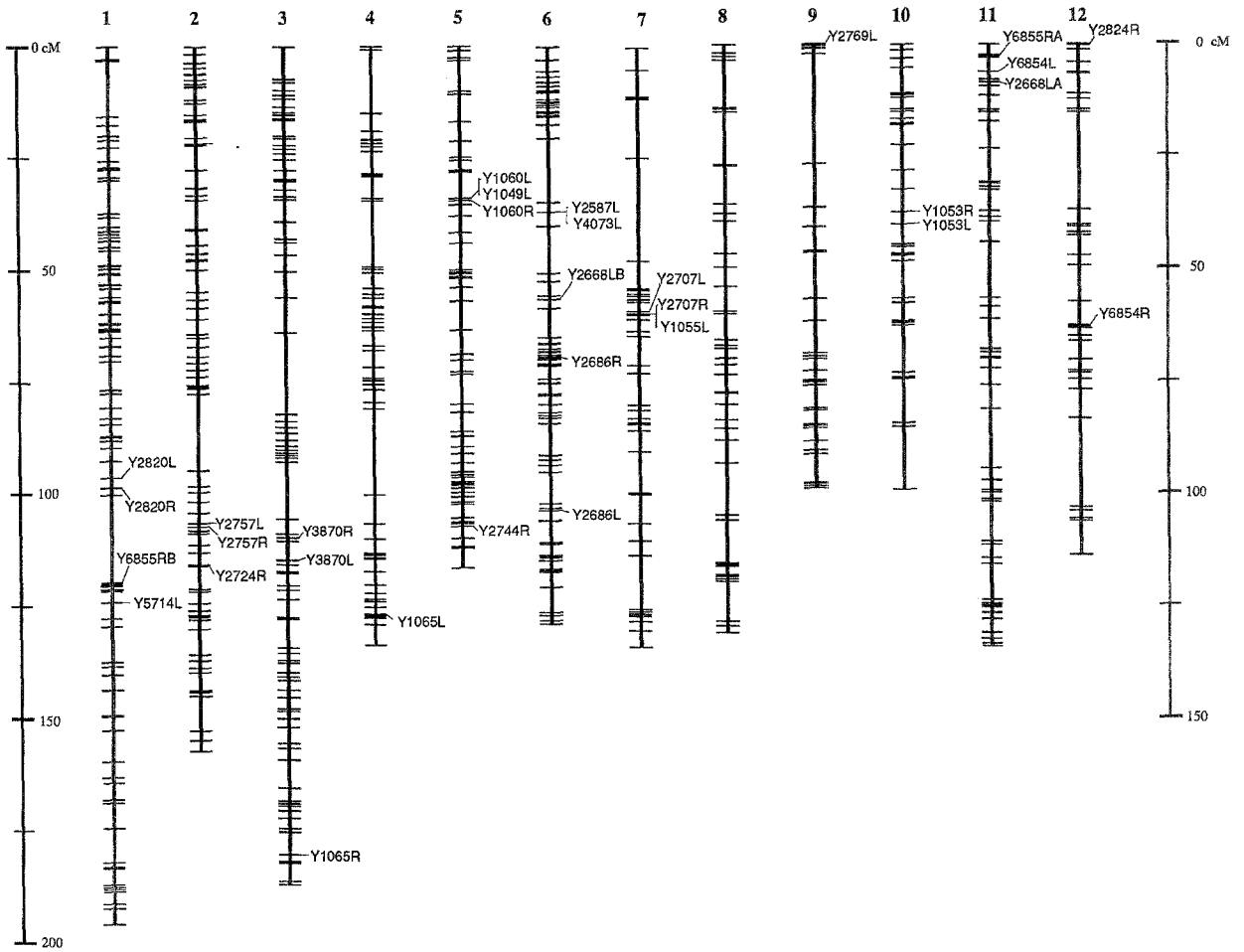

Fig. 3. YAC end clones mapped on a rice RFLP map [29]. Horizontal bars represent DNA markers. R and L following the clone names represent the right and left arm of the YAC clones, respectively. A and B represent different loci detected using the same probe. All calculations were done by MAPMAKER software; cM is expressed by the Kosambi function.

analyses, genetic engineering and breeding of all cereal plants. In the present study we were able to construct a YAC library with an average YAC size of 350 kb and a maximum size of more than 1 Mb (Figs. 1 and 2). This library covers six times of rice haploid genome (4×10^8 bp) with about 7000 clones.

For the construction of our YAC library, we extracted rice DNA from cultured cells. The use of cultured cells is advantageous, because it is easy to prepare a lot of intact protoplasts needed for the extraction of high-molecular-weight DNA. It has been pointed out that DNA of cultured dedifferentiated cells might be mutated or rearranged [15]. In cultured cells, however, regions of mutation or rearrangements in DNA seem to differ from cell to cell. Even if we use DNA extracted from normally grown plant cells, coligation causing chimeric YACs cannot be avoided. Therefore, it seems more practical to use cultured cells and eliminate chimeric or rearranged clones after examining their structure. Actually, the results of YAC end mapping obtained through RFLP analysis showed that chimerism or rearrangements of YACs were not so frequent in our *Eco* RI library (Table 1). In addition, for more detailed analysis of DNA structures, we can use other, shorter DNA clones, such as cosmids,

Table 2. The number of YAC clones isolated by RFLP markers.

DNA marker[1]	Chromosome number	Number of isolated YAC clones
G124B	4	9
G264	4	2
G267	4	5
G30	6	5
G122	6	10
G329	6	1
C226A	6	3
C226B	7	4
G44	11	1
Total		40

[1] See Nagamura *et al.* [29].

cDNA clones and genomic DNA from normal plant cells in order to examine the problems of rearrangement and co-ligation derived from cell culture and largeness of DNA.

In using the current YAC library for physical mapping and gene isolation, chimeric YACs complicate physical mapping and chromosome walking. To estimate chimera frequency of our YAC library, we tried to map both end fragments of YACs by RFLP mapping. If both end fragments of a YAC were mapped on the same region within 1 or 2 cM, the YAC should be colinear. In total 32% of YAC ends could be mapped. The remaining ends could not be mapped because of repetitiveness of the sequences (22%), because of no RFLPs with the used endonucleases (12%), or because of too weak hybridization pattern to identify polymorphic bands (34%). We used adaptor mediated PCR to amplify YAC-end fragments. Therefore the fragments shorter than 300 bp might be produced and could not be labelled with the ECL direct labelling system.

In YACs with *Eco* RI digests, most chimeras were found in YACs longer than 400 kb, consisting of about 15% of the library. Most *Eco* RI YACs shorter than 400 kb appeared to carry colinear fragments as shown in Table 1. It is suggested that chimeric YACs in the *Eco* RI YACs resulted from ligation or fusion of DNA fragments 200–300 kb in length, as described by Selleri *et al.* [33].

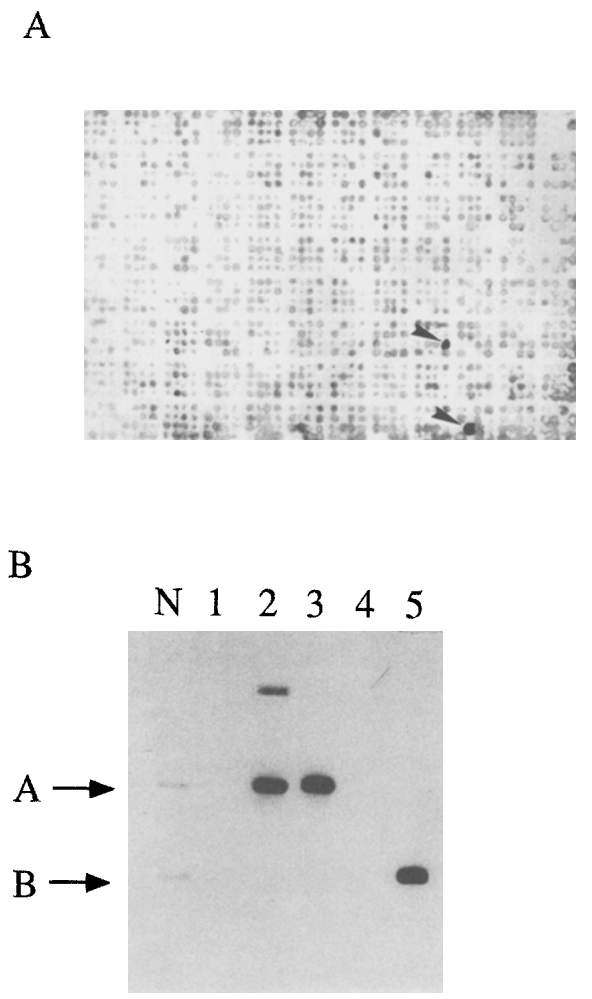

Fig. 4. A result of YAC screening by colony hybridization and Southern blot analysis. A. An autoradiogram image of YAC high-density replica filter hybridized with C226 probe. Colony hybridizations were done with the ECL system (Amersham). A typical filter out of five covering six genome equivalents is shown. Arrow heads represent positive clones, and slight signals were background. B. Southern blot analysis of candidate YAC DNAs with C226 probe. Lanes: N, rice cv. Nipponbare; 1, Y3370; 2, Y3230; 3, Y4404; 4, YN955; 5, Y2915. 'A' represents the band mapped on C226A locus of chromosome 6. The band 'B' was mapped on C226B locus of chromosome 7. The A and B bands indicate that lanes 2 and 3 are the YACs carrying C226A sequence on chromosome 6 and that lane 5 is the YAC possessing C226B sequence on chromosome 7. The artifacts in colony hybridization could be eliminated by no signals as in lanes 1 and 4.

In the *Not* I YACs co-ligated fragments might be enriched by the second size fractionation which

retained mainly fragments larger than 300 kb. On the other hand, in rice most *Not* I digests before size fractionation were smaller than 200 kb.

Our results indicated that in the *Eco* RI YACs, YACs shorter than 400 kb are rarely chimeric, but that *Eco* RI YACs longer than 400 kb and *Not* I YACs are often chimeric. Thus, RFLP mapping of YAC end fragments can be used to check rice YAC colinearity, although laborious and time-consuming. However, chimeric clones would be eliminated in the process of constructing YAC contigs by replacing colinear clones selected with the same DNA markers. Therefore, it might be important to isolate multiple YAC clones for each DNA marker on the dense linkage map.

It is presumed that the total genetic distance of rice genome is about 1600 cM [29] and the haploid genome size is ca. 400 Mb [2]. The physical distance per cM is inferred to be 250 kb on average. However, it is well known that recombination frequency is not constant along the chromosomes. For example, in the distal region of chromosome arms and in heterochromatin, physical distance is longer than the average value [15]. As shown in Table 1 and Fig. 3, the physical distance per cM varied from ca; 120 kb to 1000 kb among six YACs. Thus the genetic distance estimated from the RFLP map coincided only roughly with the inferred average physical distance, and it seems that the recombination values are different depending on the region of chromosome. Both end fragments of Y2707 (about 500 kb) were mapped at 0.5 cM apart on the marker condensed region of chromosome 7, where we have eleven markers within 0.7 cM. These markers were located close together in the linkage map, but might be located far apart in the physical map. This region of chromosome 7 may have suppressed recombination and might contain a centromere. The chromosomal locations of YACs mapped on the RFLP linkage map have not yet been identified by chromosome mapping. To know the location of YAC or DNA clones on chromosomes, it would be very interesting and important to elucidate the correlation between recombination frequency and chromosomal position. Advances in physical maps will clarify the probability of recombination in meiosis in various chromosome regions.

The usefulness of libraries depends on how easily one can isolate the clones carrying DNA markers. We could easily and reproducibly screen YAC clones by RFLP marker sequences with high-density colony hybridization and ECL system. DNAs of candidate YAC clones were digested with the endonucleases used for RFLP analysis of DNA markers, and blot-analyzed with those markers. The same signals of candidate YACs with Nipponbare DNA indicated that the YACs derived from the same loci as the RFLP markers. We could isolate YAC clones located on different chromosome positions even using probes of multiple loci, as shown in Fig. 4B. In other cases, the loci of only some of the DNA bands could be determined due to lack of polymorphism between cvs. Nipponbare and Kasalath, parents of the mapping population. Isolation of YAC clones containing not only loci of polymorphic bands but also loci of non-polymorphic bands is very useful for further physical mapping, because we can make YAC contigs with these bands and decide the position of non-polymorphic bands on the physical map by overlapping hybridization signals of other RFLP markers. The number of YAC clones with RFLP marker sequences isolated from this library are shown in Table 2. These show that these *Eco* RI YAC clones can be used to isolate YACs harboring almost all RFLP markers.

For construction of ordered rice genomic library it seems most effective to isolate YAC clones with DNA markers on the genetic map, first to make contigs and next to fill-in gaps between contigs by chromosome walking. Chromosome walking is very time- and money-consuming work. Therefore, it is important that DNA markers are mapped densely enough on the linkage map so as to isolate YACs carrying more than two DNA markers. Then, many YAC contigs can be constructed rapidly and easily without chromosome walks. More than 800 DNA markers have been mapped on rice linkage map in our laboratory (Nagamura *et al.* [29]), and soon we will have two thousand markers on the map. Since YAC

clones harbor on average 350 kb inserts in the present library, YACs isolated with two thousand DNA markers are expected to cover near 400 Mb, spanning over 90% of the rice haploid genome. We have already isolated independent YAC clones carrying two DNA markers 0.3, 0.5, 0.8, 1.0 and 1.3 cM apart (unpublished data). Thus, it is expected that many DNA markers can be linked to YACs without chromosome walking.

We have for the first time constructed a high-quality rice YAC library suitable for physical mapping. Our YAC clones are freely available for academic research as and when requested. We have already started isolation and ordering of YACs with DNA markers for the formation of YAC contigs along the RFLP map. These ordered YAC contigs should be very useful for further detailed genome analysis and map-based cloning.

Acknowledgement

We thank Takashi Imai for expert advice and kindly providing with *Saccharomyces cerevisiae* AB1380, Yuji Kohara for kind advice on the preparation of YAC high-density filters, Ei-ichi Soeda and Kazunari Yokoyama for encouragement, Kaetsu Kobayashi, Takuji Sasaki, Yuichi Katayose and Akio Miyao for valuable discussions and useful advice, Ilkka Havukkala and Baltazar A. Antonio for helpful comments on the manuscript. We are grateful to Perry J. Gustafson for kind suggestions. pYAC4 and pYAC55 were provided from GenBank, Tsukuba Life Science Center, Institute of Physical and Chemical Research (RIKEN). This work was supported by the Ministry of Agriculture, Forestries and Fisheries, Japan and by the Japan Racing Association.

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