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Saturation mapping with subclones of YACs: DNA marker production targeting the rice blast disease resistance gene, *Pi-b*

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Abstract Saturation mapping of a very small genomic region is indispensable for map-based cloning. We applied a method based on sub-cloning and the Southern-hybridization technique for generating RFLP markers directly from yeast artificial chromosomes (YACs). Two YACs overlapping each other and covering the locus of the rice blast resistance gene, *Pi-b*, were used to construct a plasmid sublibrary. Rice-specific and single-copy clones suitable as probes for RFLP analysis were selected from this sub-library by hybridization to the blots of digested DNAs of rice, YACs, and yeast. As a result, 22 markers were produced within a small chromosomal region including *Pi-b*. This case study shows that overlapping YACs known to cover the gene of interest are very useful in fine-scale physical mapping leading to map-based cloning of the target gene.

Key words Saturation mapping · Yeast artificial chromosome (YAC) · Rice · *Pi-b* · Blast disease resistance

Introduction

For map-based gene cloning, DNA markers flanking both sides of the target gene are indispensable. If such markers are physically very close to the target gene, then the small genomic fragment holding that gene can be screened using these markers as probes. To be effective, the distance between the markers should be less than the average insert length of a conventional library, such as a cosmid library. This technique, known as "chromosome landing", is a sophisticated strategy for the map-based cloning of important genes (Tanksley et al. 1995).

For detecting DNAs from limited chromosomal regions, the technique of bulked segregant analysis (Michelmore et al. 1991) has been established. Previously we produced many RAPD markers by this method at an interval of approximately 30 cM and tagged the rice photoperiod-sensitivity gene on chromosome 6 (Monna et al. 1994). Although the target region can be set arbitrarily by the bulked method, a prohibitively large number of screenings would be required to detect polymorphisms derived from a very small region. Therefore, a new approach is necessary for producing markers in a very small interval of a few cM or several hundred kilobase pairs. Attempts to produce microsatellite markers at chosen loci by shearing and subcloning yeast artificial chromosome (YAC) DNA have been reported for both the human genome (Cornelis et al. 1992; de Souza et al. 1994; Brown et al. 1995) and fungal genomes (Chen et al. 1995) .

The rice blast disease resistance gene *Pi-b* is one of the agronomically important genes of rice. This gene was discovered in *indica* cultivars and localized on the end of chromosome 2 (Yokoo et al. 1978). Near-isogenic lines (NILs) of *japonica* cultivars carrying *Pi-b* segments introduced from resistant cultivars have been developed (Yokoo et al. 1978). Some molecular markers tightly linked to *Pi-b* have been determined using the cross of BL-1, the NIL, and a susceptible *japonica* cv, Aichi Asahi (Miyamoto et al. 1996). There are also several RFLP markers developed in this region of the Nipponbare-Kasalath high-density genetic map (Kurata et al. 1994b). However, map-based cloning of this gene requires more molecular markers that flank both sides of, and are physically close to, the gene.

In the present study we tried to use sub-cloned libraries of YAC clones which cover the *Pi-b* gene as a source of hybridization probes, and generated a number of RFLP markers tightly linked to this gene. The physical distribu-

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tion of generated RFLP markers in this region has been also investigated.

Materials and methods

Plant materials

Tohoku IL9 is an isogenic line of the *japonica* cultivar Sasanishiki having the *Pi-b* segment derived from Engkatek and was developed by Miyagi Prefecture Furukawa Agricultural Experimental Station. DNAs of the *japonica* cultivar Nipponbare, the *indica* cultivar Kasalath and 186 F_2 plants, as well as Sasanishiki and Tohoku IL9, were extracted from green leaves by the CTAB method (Murray and Thompson 1980).

YAC clone determination

Linkage mapping using RFLP markers revealed that *Pi-b* is located between two RFLP markers, C2782B and C379, that were established on the Nipponbare Kasalath high-density linkage map (Hayasaka et al., unpublished data, reported at 87th meeting of Japanese Society of Breeding, 1995, Ibaraki, Japan). A YAC library was constructed from Nipponbare DNA, and YAC clones covering this region were screened with C2782B, C379, and other RFLP markers established within this interval, following the method reported previously (Umehara et al. 1995). Of these YACs, we selected Y3802 and Y6791 that overlap each other and have appropriate insert sizes (290 and 320 kb, respectively) for pulsed-field gel electrophoresis (PFGE) separation.

Purification of YAC insert DNA by PFGE

Agarose plugs containing high-molecular-weight DNAs of Y3802 and Y6791 were prepared by the method of Imai et al.(1990). DNAs were electrophoresed on a 1% low-melting-point agarose gel in a contour-clamped homogeneous electric field (CHEF) apparatus for 22 h, at 6 V/cm by sequential switching from 20 s to 50 s, in 0.5×TBE at 14°C. For YAC DNA extraction and purification, we followed the method of Vaudin et al.(1995). Starting with 80 plugs (four gels), 200 μl of DNA solution was obtained for each of Y3802 and Y6791.

Construction of the sub-library of YACs

Equal amounts of each purified DNA solution of Y3802 and Y6791 were mixed and partially digested with *Sau*3AI. The resulting fragments were ligated to the *Bam*HI site of pBluescriptII SK+ and transformed *E. coli* NM522.

Transformants with an insert length of more than 200 bp were selected by PCR with the synthesized primers M4 (5′-GTTTTCCCAGTCACGACGTT-3′) and RV(5′-CAGGAAA CAGCTATGACCAT-3′) using a cell suspension as the template. Reaction mixtures were incubated at 94°C for 1 min before 25 cycles of 94°C-1 min, 60°C-1 min and 72°C-2 min. Amplification of insert DNAs for preparing hybridization probes was performed under the same conditions.

Screening sub-cloned fragments for RFLP analysis

DNAs of Nipponbare, Y3802, Y6792 and yeast were digested with *Sau*3AI, electrophoresed in 1.5% agarose gels and blotted onto nylon-membrane filters (Boehringer Mannheim). Insert DNA of each clone was amplified as described above, labelled using an ECL direct labelling kit (Amersham) and hybridized to the blots. Clones that gave clear bands on the Nipponbare and YAC lanes, but not on the yeast lane, were considered as rice-specific single-copy clones and were used for linkage analysis.

Linkage analysis

Parental polymorphism screening and linkage analysis using the Nipponbare-Kasalath F_2 population were performed following the method reported previously by Kurata et al. (1994b). The detection of polymorphisms between Sasanishiki and Tohoku IL9 was also performed following the same procedure.

Determination of the physical distribution of mapped clones

Restriction maps of YAC DNA fragments were constructed substantially following the methods of Imai et al. (1990). High-molecular-weight DNAs were digested in agarose plugs with the restriction endonucleases *Not*I, *Nru*I, *Mlu*I and by combinations of *Nru*I-*Mlu*I and *Not*I-*Mlu*I. The resulting DNAs were separated by PFGE at 6 V/cm for 16 h by sequential switching from 10 s to 20 s and transferred to nylon-membrane filters. Probes prepared from mapped clones and left or right arms of the YAC vector sequence were then hybridized to the blots.

Sequence determination and homology search

Nucleotide sequences of mapped clones were determined by single run sequencing from one end using the universal, 21M13 Dye, primer (Perkin Elmer) and the Ampli*Taq* DNA polymerase CS+ DNA sequencing kit (Perkin Elmer) according to the manufacturer's instructions. Sequencing was performed on an automated fluorescent DNA sequencer (Model 373A, Applied Biosystems).

The BLASTN algorithm was used for searching identical sequences from approximately 20 000 cDNA sequences already determined by the Rice Genome Research Program (RGP). The homology search program Mas Per Search was used through the DNA Information and Stock Center (NIAR, Japan) for searching the homology of nucleotide sequences and amino-acid sequences. The DDBJ, EMBL and GenBank public databases were used for nucleotide sequences, and PIR, SWISS-PROT, GenPept and PDB for amino-acid sequences.

Results

Screening sub-cloned fragments suitable for RFLP analysis

The results of the hybridization screening to eliminate yeast-derived clones and rice-derived multi-copy clones are described below and shown in Fig. 1. Out of 129 clones tested, the number of rice-specific single-copy clones was 47 (37%), the number of rice-specific multi-copy clones was 33 (25%), and the number of yeast-specific clones was 49 (38%). Rice-specific single-copy clones were divided into three groups according to the hybridization pattern to the blots of rice, YACs, and yeast DNAs: (1) hybridizing with both Y3802 and Y6791, (2) hybridizing with Y3802 but not with Y6791 and (3) hybridizing with Y6791 but not with Y3802 (Fig. 1). As this pattern roughly indicated the mapped position of the clone, it was decided at this step whether the clone was, or was not, useful for further analysis. One chimeric clone, G7019, having a full insert length of 2100 bp with two Southern bands was detected (1500 bp and 600 bp). The large fragment was present only on Y3802 whereas the small one was on both Y3802 and Y6792 (Fig. 1). There were no YAC vector-specific clones, which would have given signals only in YAC-clone lanes.

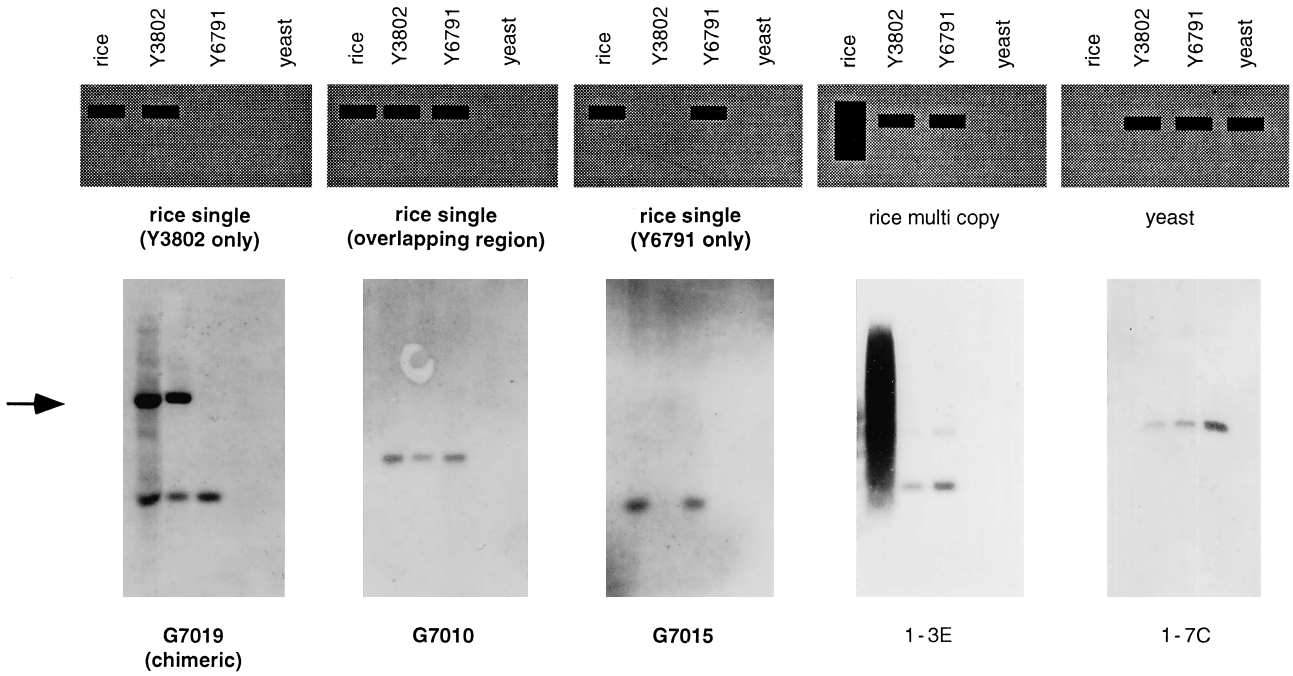

Fig. 1 Predicted patterns (above) and detected Southern-hybridization images (below) obtained in the screening of rice-specific single-copy clones. 1-3E and 1-7C were a multi-copy clone and a yeast-specific clone, respectively, and were not used for further analysis. G7010, G7015 and G7019 were examples of rice-specific single-copy clones and were used as RFLP probes

Linkage mapping of genomic clones generated in this study

Forty-seven rice-specific single-copy clones were used for parental RFLP screening, and 22 showed clear polymorphisms between Nipponbare and Kasalath. The remaining 25 included monomorphic clones, clones smearing with a single major band, and clones with comparatively weak signals, which were considered inappropriate for further analysis.

Twenty two clones, named G7009–G7030, were mapped and established as new markers. Table 1 shows the insert length, the mapped position on the Nipponbare-Kasalath map, and the enzyme(s) giving RFLPs between Nipponbare and Kasalath, and also between Sasanishiki and Tohoku IL9. The insert length of each clone was determined by PCR with universal primers. The sizes of Southern hybridization band(s) were estimated from the results of the first screening.

Table 1 [a, b] Restriction enzymes which revealed polymorphisms between Nipponbare and Kasalath ([a]) or Sasanishiki and Tohoku IL9 ([b]). Italics indicate enzymes used for linkage analysis. Abbreviation: *Ba*; *Bam*HI, *H*; *Hin*dIII, *D*; *Dra*I, *EI*; *Eco*RI, *EV*; *Eco*RV, *K*; *Kpn*I, *A*; *Apa*I, *Bg*; *Bgl*II. Polymorphisms are co-dominant unless especially indicated. [c] The loci of the markers determined on Nipponbare/Kasalath high-density linkage map are shown as the distances from upper end of chromosome 2 of our linkage map

Marker	Size (bp)	Polymorphic enzymes between N/K[a]	Polymorphic enzymes between S/T9[b]	Locus (from end)[c]
G7016	423	*EV*, EI	Monomorphic	1.6 cM
G7019	2100	*A*, Bg, EI	Bg, A	1.6
G7022	600	*D*	D	2.7
G7013	342	*Bg* (dominant)	Bg (dominant)	2.7
G7018	1100	*EV*, A, EI	EI	2.7
G7027	700	*A*	Monomorphic	2.7
G7028	378	*A*	Monomorphic	2.7
G7029	1250	*A*	Monomorphic	2.7
G7010	243	*Ba*, D, K	Ba; D, K	3.8
G7021	297	*D*, Bg	Ba, D, K	3.8
G7023	1300	*Bg*, EV, D, K	Bg, K	3.8
G7030	750	*H*	A (dominant)	3.8
G7026	334	*EI*, EV	H	4.1
G7012	600	*H*	H, A (dominant)	4.4
G7020	229	*Ba* (dominant), Bg, EV, A, EI	Ba, Bg, EV, EI	4.4
G7027	262	*Ba*, Bg, D	Ba, Bg, EV, A, D, EI	4.4
G7011	159	*EV*	Monomorphic	4.7
G7014	250	*H*, Bg	H, Bg (dominant)	4.7
G7017	1000	*EI*, EV	EI	4.7
G7025	275	*H*, EV, D, EI	H, D	4.7
G7009	1350	*Ba*	Ba, EV	5.2
G7015	423	*Ba*	Ba	5.2

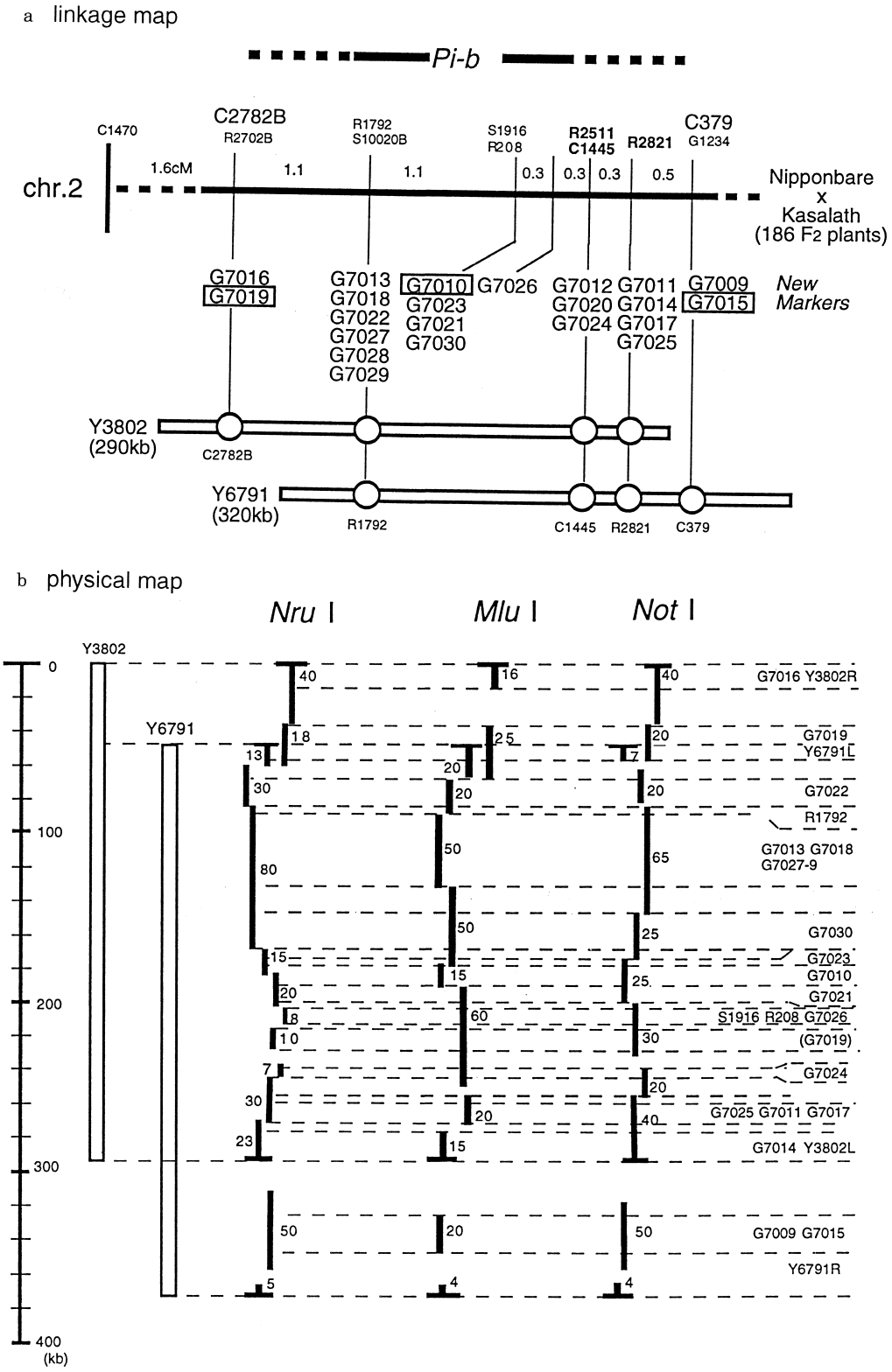

Fig. 2a Genetic location of 22 markers obtained in this study (G7009–G7030). The candidate region of *Pi-b* was determined between RFLP markers C2782B and C379 using the F_2 population of Sasanishiki/Tohoku IL9, and is indicated above the linkage maps by a *thick horizontal line* (Hayasaka et al., see Materials and methods). *Open horizontal bars* below the linkage map represent two overlapping YACs. RFLP markers which hybridized to each YAC at the selection of YAC clones are denoted by *open circles* (Umehara et al., 1995). Note that markers G7019, G7010 and G7015 (shown as *open squares*) were mapped onto reasonable positions predicted by the results of screening (see Fig. 1). **b** Presumed physical distribution of DNA markers obtained in this study. *Open vertical bars* represent two overlapping YACs (Y3802 and Y6791). *Short vertical lines* indicate *Not*I, *Mlu*I and *Nru*I fragments detected by Southern hybridization of each marker to the blots of digested and PFGE-separated YAC DNAs. Fragments with 'Y3802L(R)' and 'Y6791L(R)' were detected by using PCR-amplified sequences of YAC, left or right arm, as probes. The order of the fragments was determined according to the results of linkage analysis (Fig. 2a) and the results of double digestion using '*Not*I and *Mlu*I' and '*Nru*I and *Mlu*I'. The markers which landed on each fragment are listed to the right, separated by *horizontal dotted lines* indicating the restriction sites identified by double-digestion. The calculated sizes of YACs are smaller than expected from the first PFGE. This suggests that there are more restricted fragments from which none of these markers are derived

The chimeric clone G7019 described above showed two major bands during RFLP analysis; one was polymorphic and the other was monomorphic. The polymorphic band was mapped to the position shown in Fig. 2a, indicating that the 1500-bp fragment was derived from this locus.

All the markers except G7013 and G7020 were mapped as co-dominant RFLPs markers. G7013 and G7020 hybridized to both the Nipponbare and Kasalath allele, but mapped as dominant markers because the 'Kasalath' band could not be distinguished from weak signals of the same

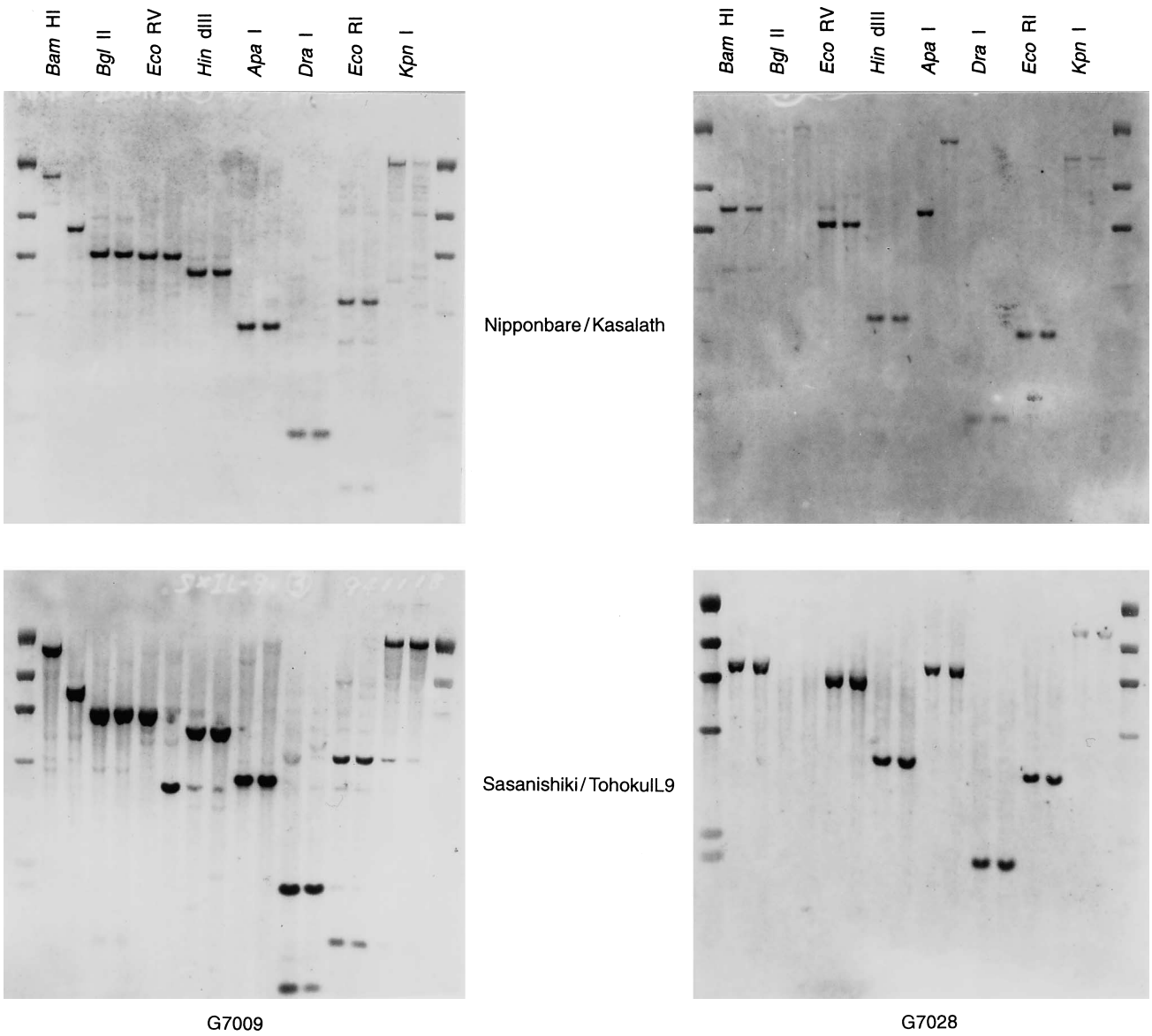

Fig. 3 Examples of parental RFLP analyses using Nipponbare-Kasalath (N-K, above) and Sasanishiki-Tohoku IL9 (S-T9, below). G7009 shows the same polymorphisms as N-K (*Bam*HI) and an additional one (*Eco*RV) with S-T9. G7028 polymorphism between N-K in the *Apa*I lanes is not observed with S-T9

size band observed in the Nipponbare allele. Although all the clones obtained were mapped into the target interval (Fig. 2a), all markers except G7026 were mapped to positions of previously established markers. The two largest gap-regions of 1.1 cM remained (Fig. 2a).

Polymorphisms between Sasanishiki and Tohoku IL9

The preparation of Southern-blot filters of Sasanishiki and Tohoku IL9 DNA, together with hybridization and detection, were performed in the same way as the parental RFLP between Nipponbare and Kasalath, using eight six-base cutting enzymes. Seventeen out of the twenty two clones (77%) gave polymorphisms between Sasanishiki and Tohoku IL9, and could be used for analyzing linkage with *Pi-b* (Fig. 3). The remaining five markers (G7016, G7026–G7029) were monomorphic between Sasanishiki and Tohoku IL9 with these eight enzymes.

Homology search of genomic clones among known cDNA sequences

Out of the 22 clones, there were three expressed genomic sequences identical to cDNA sequences compiled by our large-scale cDNA analysis program: G7020 and R2511 (D24756), G7025 and R2821 (D24942), and G7019 and C1445 (D15870). R2511 and R2821 were cDNA clones prepared from roots, and C1445 was from callus. These three cDNA clones were already established as RFLP

markers (Kurata et al. 1994b). As shown in Fig. 2a, G7020 and R2511 mapped to the same position, as did G7025 and R2821. The G7019 and C1445 loci were separated by approximately 3 cM, which is reasonable because G7019 is a chimeric clone and the 600-bp fragment that gave hybridization signals with both Y3802 and Y6791 is presumed to be from the C1445 locus. Interestingly, parental RFLP patterns were similar, but not exactly the same, between G7020 and R2511 and between G7025 and R2821, possibly reflecting the structure of cDNA sequences made up of exons which are physically apart in the genomic sequences.

The overlapping region of G7020 and R2511 was homologous to the potassium-channel beta-subunit protein of *Arabidopsis* (L40498). This is the second discovery of this sequence in plants, and in *Arabidopsis* this gene codes for a 38.4-kDa polypeptide (Tang et al. 1995). C1445 was homologous to the conserved domain IV of auxin-induced proteins of *Arabidopsis*, soybean and other plants (Abel et al. 1994) while G7019 contains a 130-bp intron. R2821 is homologous to the myosin of *Arabidopsis* (Kinkema and Schiefelbein 1994, Z28389), and G7025 contains the 93-bp intron that is highly homologous to the yeast SCD26 protein (dosage-dependent suppressor of the cell-cycle mutation cdc26, X17118) (Dascher and Kuntzel 1989). The other 19 clones do not have any sequences similar to our cDNAs.

Physical distribution of mapped fragments

The main purpose of this study is to develop a local, very fine-scale, genetic and physical map. Linkage analysis has localized the new markers in clusters, raising concern that the sub-clones were not obtained evenly from YAC fragments. Therefore, we tried to construct a physical map of this region using the restriction endonucleases *Not*I, *Nru*I and *Mlu*I and investigated the physical distribution of the mapped fragments. According to the Southern-hybridization patterns and the linkage map, the physical distribution of markers obtained in this study is presumed to be as shown in Fig. 2b. As the total size of the detected fragments is smaller than expected from the original YAC size, there should be some restriction fragments on which no marker landed. The physical distance of the 1.1-cM gap on the linkage map turned out to be less than 80-kb, as G7018 (and G7013, G7027-9) and G7030 landed on the same 80-kb *Nru*I fragment. Also, some markers that were mapped to the same position were physically localized on different restriction fragments.

Discussion

Construction of a fine physical map for positional cloning requires a number of molecular markers in a particular interval. The original aim of the present study was to provide a number of polymorphic DNA markers for more precise linkage analysis of the *Pi-b* region. To achieve this, we constructed a sub-library of YAC clones covering the region and used it as a source of RFLP probes.

A single PFGE followed by excision and beta-agarase digestion for YAC DNA purification cannot remove yeast chromosomal DNA completely. In fact, 38% of the clones tested carried yeast-derived sequences. Although this percentage seems rather large, it may be possible by controlling the PFGE conditions to separate YACs and yeast chromosomes more clearly. Vaudin et al. (1995) performed two rounds of PFGE and succeeded in reducing yeast DNA contamination to under 10%. However, considering the additional time and techniques required for a second PFGE, and as the screening is rather easy in our procedure, the purity of YAC DNA from a single PFGE appears tolerable.

Selection of "rice or yeast" and "single- or multi-copy" clones can be done simultaneously by Southern hybridization-based screening of sub-clones. Although we used only two overlapping YACs, more informative results can be obtained if additional YACs overlapping each other are available. Thus it is possible to decide at this step whether the clone is in or out of the target region, and therefore if further analysis of it is necessary.

Twenty two markers were generated within a small chromosomal region including *Pi-b*. Linkage analysis localized all these markers except one (G7026) to the same loci as already existing markers; and two gaps of 1.1 cM each still remained (Fig. 2a). This is a serious problem that may occur in any region of the genome to be analyzed. In this case, however, restriction map construction revealed that a 80-kb *Nru*I fragment contains the 1.1-cM gap region. The existence of a recombination hot spot is considered to be one of the possible reasons producing a gap in such a small interval, but more genetical and molecular biological information will be required to solve this problem.

Some markers mapped to the same position landed on different restriction fragments that are physically apart. On the other hand, G7011, G7017 and G7025 make a cluster on both the genetic map (Fig. 2a) and the physical map (Fig. 2b).

The sequences of these clones were compared with roughly 20 000 cDNAs determined by the RGP and 3 out of the 22 were identical in expressed sequences. No base substitution or deletion was observed between these sequences. A comparison between the genomic sequences of *C. elegans* determined directly from cosmid clones and from the M13 sub-library constructed with YAC DNA indicated that the original genomic sequence is well preserved in YAC clones (Vaudin et al. 1995).

Out of the 22 RFLP markers generated from Nipponbare genomic fragments, 17 showed RFLPs between Sasanishiki (rice blast susceptible cultivar) and Tohoku IL9 (rice blast resistant NIL of Sasanishiki) and thereby were useful for the linkage analysis of *Pi-b*. This result shows that we can use the established linkage maps and the ordered large insert DNA clones for producing RFLP markers between arbitrary selected rice cultivars. As the synteny between rice and other cereal crops, for example, wheat, maize, sorghum, sugar cane, millet and barley (Ku-

rata et al. 1994a; Dunford et al. 1995; Killian et al. 1995; Moore et al. 1995a; Moore et al. 1995b), has been analyzed the procedure and results described here can be applied to any plant species if the target region shows similarity with rice.

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