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Construction of rice chromosome specific DNA libraries using chromosome microdissection technique and its application for mapping

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The DNA libraries derived from the entire chromosomes 1, 4, 7 and 9, and the extra chromosomes of acrotrisomics (Acro5a) were constructed using the chromosome microdissection technique. Fluorescence *in situ* hybridization (FISH) was used to identify the origins of the constructed DNA libraries. DNA libraries from the entire chromosomes 1, 4 and 9 would be available for the identification of chromosome on FISH. In addition, the origin of DNA libraries from the entire chromosome 7 and the extra chromosomes of Acro5a were identified by dot blot hybridization. One hundred sixty and 587 clones were obtained from DNA libraries of the entire chromosomes 1 and 4, respectively. Sixty-three of these clones were surveyed for polymorphism among six varieties. Forty-three unique clones, 35 clones for polymorphism and 20 multicopy clones were obtained. Two of 160 clones and three of 587 clones were mapped on RFLP linkage map using recombinant inbred lines.

INTRODUCTION

Chromosome microdissection technique has been developed for genome DNA analysis of specific chromosome regions. This technique is used for construction of DNA libraries derived from target regions on chromosome preparation. The first chromosome microdissection technique was used in *Drosophila* (Scalenghe *et al.*, 1981), then mouse (Röhme *et al.*, 1984) and human (Lüdecke *et al.*, 1989; Lüdecke *et al.*, 1990; Kao and Yu, 1991; Hadano *et al.*, 1991; Hirota *et al.*, 1992). DNA libraries obtained using this technique can be utilized as probes for chromosome painting in fluorescence *in situ* hybridization (FISH) (Ohta *et al.*, 1993).

In comparison to genomic DNA analysis, chromosome microdissection technique is better because it is possible to obtain DNA from special regions on chromosome preparation. Since a number of clones could be derived from special chromosome regions, DNA relative to target genes is selected in high frequency. DNA fragments could be obtained from the centromere, telomere, interchanged point and breakpoints directly, hence, utilizing their DNA fragments will lead to contribute analysis of mechanism and construction for centromere, telomere and chromosome aberration.

Fukui *et al.* (1992) reported that chromosome microdissection technique might be used in rice and barley. Construction of chromosome region specific DNA libraries was

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made in rice (Nonomura *et al.*, 1994), wheat (Vega *et al.*, 1994) and barley (Busch *et al.*, 1995, 1996).

Here, we constructed chromosome regions specific DNA libraries from the entire chromosomes and an acrocentric chromosome using microdissection technique to locate their DNA clones on RFLP linkage maps.

MATERIALS AND METHODS

Chromosome preparation for microdissection

Seeds of Japonica variety "Nipponbare" and acrotrisomics (Acro5a; Ikeda *et al.*, 1999) having an extra acrocentric chromosome for chromosome 5 were used for chromosome preparation. The acrocentric chromosomes for chromosome 5 in addition to the entire chromosomes 1, 4, 7 and 9 were targeted for microdissection. Excised roots from germinating seeds at 30 °C were pre-treated with 1.5 mM 8-oxyquinoline for 1.5–2 h at 20 °C, and were fixed with a fixative (3 methanol : 1 acetic acid) for 2 h at room temperature. Their roots were stocked in 70% ethanol at 4 °C. Chromosome preparation was based on the modified method of Kurata and Omura (1978). Stocked roots were immersed in distilled water for 30–60 min at room temperature. Root tips were treated with the enzyme solution which contained 3% cellulase (Onozuka RS), 1.5% pectolyase Y23, 7.5 mM KCl and 7.5 mM EDTA adjusted to pH 4.0 for 31–33 min at 37 °C. Treated root tips were immersed in distilled water for 30–60 min. Prometaphase chromosomes were air dried then fixed in 9 methanol : 1 acetic acid and steamed using water bath at 65 °C for 20–30 seconds. Well spread chromosomes were washed using 100% methanol and stained with 10% Giemsa solution for 2 h.

Microdissection and PCR amplification of chromosome DNA

Procedures followed those described previously in human (Hirota *et al.*, 1992; Ohta *et al.*, 1993). A fragment of chromosome was microdissected under an inverted microscope with the use of a fine glass needle, guided by micromanipulator. Collected chromosome pieces were treated with 2–3 nl of proteinase K solution (0.5 mg/ml proteinase K, 0.1% SDS, 10 mM Tris-HCl (pH 7.5), 10 mM NaCl) at 37 °C for 3 h. The extracted DNA was digested with *Sau* 3AI solution (50 U/ml *Sau* 3AI, 100 mM NaCl, 10 mM Tris -HCl (pH 7.5), 7 mM MgCl₂) at 37 °C for 4 h. The DNA fragments were amplified with the primer-linker PCR method. Ligation reaction was performed using ligation mixture (a 10 mer linker [MboL1; 5'-GATCCATGTC-3'], a 24 mer primer [NlaM1; 5'-CGGGAATTCTGGCTCTGCGACATG-3'], 350 U/ml T4 DNA ligase in ligation buffer) at 15 °C for 1 h. PCR was performed as follows: The first cycle, 73 °C (2 min), 94 °C (3 min), 62 °C (2 min), 72 °C (2 min). Followed by 30 cycles, 94 °C (2 min), 62 °C (2 min), 72 °C (2 min). Lastly at 72 °C (5 min).

Fluorescence in situ hybridization (FISH)

Procedures for FISH involved a modification of the method described previously (Ohta *et al.*, 1993). The second PCR products were used for probes in FISH, a small volume (5 ml) of the first PCR products was amplified by 12 cycles, 94 °C (2 min), 62 °C (2 min), 72 °C (2 min), in a reaction mixture containing 100 mM biotin-16-dUTP and 100 mM dTTP. The second PCR products with 10 mg sonicated salmon DNA and 0–8 mg

sonicated DNA of rice cultivar "IR24" were used for hybridization. The DNA mixture was ethanol-precipitated, resuspended in 10 ml formamide, denatured at 80 °C for 10 min, and mixed with a hybridization solution consisting of 40 mg bovine serum albumin (BSA), 4 × SSC and 20% dextran sulfate. The hybridization was performed at 37 °C for 12–18 h. The slides were washed in 50% formamide and 2 × SSC (15 min), then 2 × SSC (10 min), 1 × SSC (5 min) and 4 × SSC (5 min) each at 37 °C; incubated with 0.5 mg fluorescence isothiocyanate (FITC)-conjugated avidin with 4 × SSC and 1% BSA at 37 °C for 45–60 min; rinsed in 4 × SSC with shaking (5 min), 4 × SSC containing 0.02% Tween 20 with shaking (7 min), 4 × SSC with shaking (3 min) and 2 × SSC (30–60 sec) each at room temperature in the dark. The chromosomes were stained with 0.1–0.2 mg/ml propidium iodide (PI), and then were observed under fluorescence microscope.

Dot blot hybridization

PCR products derived from target chromosomes and genomic DNA of "Nipponbare" as control were used for dot blotting on nylon membranes. The RFLP markers labeled with (α -³²P) dCTP were utilized for Southern hybridization (Table 1). Dot signals were detected when a part of PCR products is homologous with the RFLP marker.

Table 1. RFLP clones used for identification of PCR products by Southern analysis

Chromosome	RFLP clones					
1	Npb302	Npb346	Npb359			
2	Npb42					
3	Npb48					
4	Npb49	Npb177	Npb237	Npb247	Npb311	R288
5	Npb25	Npb105	Npb208	Npb292	Npb297	R2232
6	Npb172	Npb342				
7	Npb106	Npb117	Npb338	Npb379	R1440	
8	Npb278					
9	Npb13	Npb103	Npb293			
10	Npb37					
11	Npb52	Npb181	Npb257			

The Npb clones were from Saito *et al.* (1991) and the others were from Kurata *et al.* (1994)

Characterization and polymorphism of clones

Procedures for cloning followed that described previously in human (Hirota *et al.* 1992). PCR products were digested with *Eco* RI at 37 °C for 4 h. DNA fragments were ligated to dephosphorylated *Eco* RI-cut pUC19 in reaction mixture at 15 °C for 8 h, and used for transformation. Recombinant plasmids were picked out and stocked at –80 °C. DNA clones were used for characterization and polymorphism using four Japonica varieties; "Nipponbare", "Taichung 65", "Asominori" and "Kinmaze", and two Indica varieties; "IR24" and "DV85". The genomic DNA of six varieties extracted by CTAB method (Rogers and Bendich 1988) were digested with six enzymes (*Bam* HI, *Bgl* II, *Dra* I, *Eco* RI, *Eco* RV, *Hin* dIII). The digested DNA was transferred to a nylon membrane

after electrophoresis. DNA clones labeled with (α - 32 P) dCTP were utilized for probes in Southern hybridization, as described by McCouch *et al.* (1988).

Mapping of clones

Five DNA clones detected as polymorphism between Asominori and IR24 were utilized for mapping using 71 recombinant inbred lines (RILs) derived from their crosses. Five clones, 1W26 and 1W49 from the entire chromosome 1, 4W203, 4W206 and 4W221 from the entire chromosome 4 were mapped based on RFLP linkage map established by Tsunematsu *et al.* (1996) using RILs. Linkage analysis was performed using MAPMAKER (Lander *et al.* 1987) and Map Manager (Manly 1993) as described by Tsunematsu *et al.* (1996).

RESULTS AND DISCUSSION

PCR products and FISH

The results showed that chromosome region specific DNA libraries could be constructed within the 200 to 500 bp DNA fragments (Fig. 1). Using FISH with suppression to non-specific regions, chromosomes 1, 4 and 9 are shown (Fig. 2b-d). Chromosome 1 is the longest chromosome, chromosome 4 is a subtelocentric chromosome and chromosome 9 is a nucleolar chromosome. Using FISH with non-suppression, specific chromosome regions plus other chromosome regions were painted. These results suggest that non-specific DNA regions share to each chromosome in rice. Non-specific DNA regions were believed to exist in heterochromatin regions because the painted

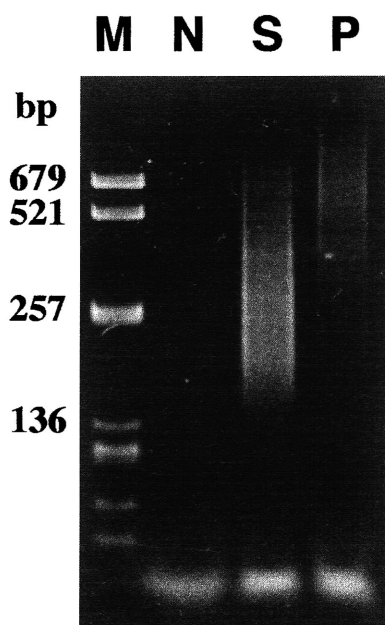


Fig. 1. PCR product dissected from the entire chromosome 4 on polyacrylamide gel. M is pUC 19/*Alu* I size marker. N is PCR product without DNA fragment. S is PCR product with sample DNA fragment. P is PCR product with rice DNA fragment.

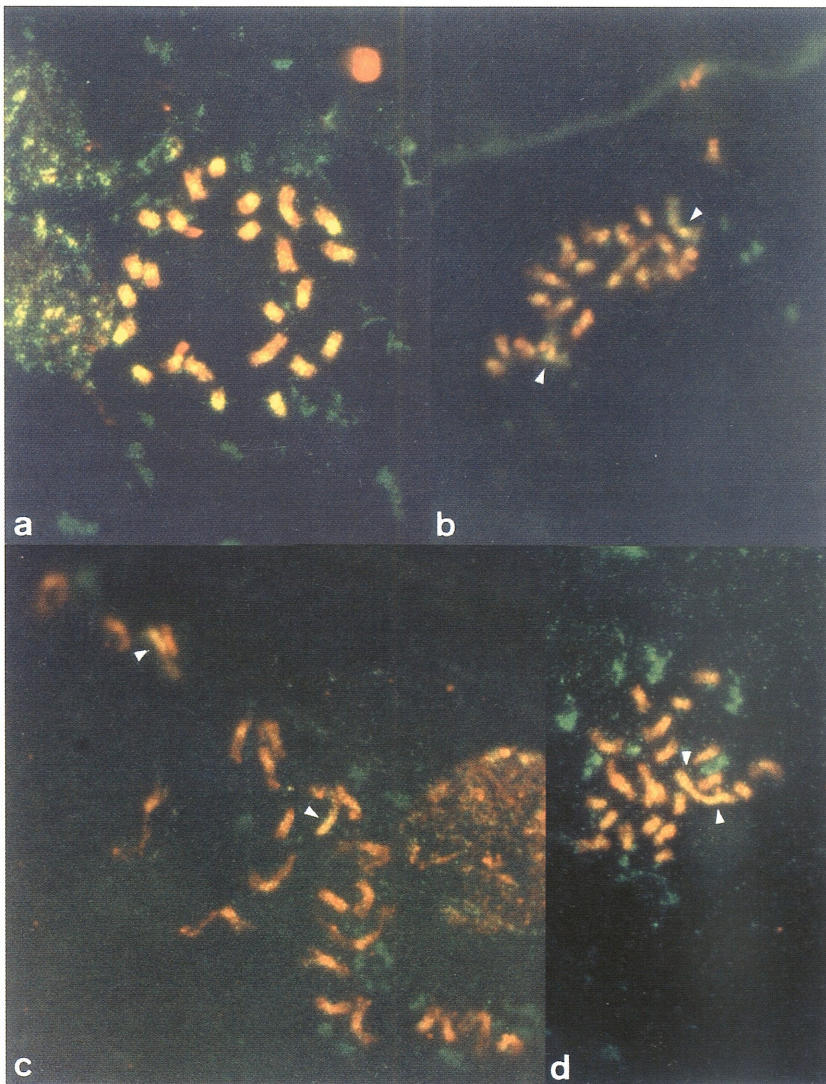


Fig. 2. a-d. Fluorescence *in situ* hybridization (FISH) analysis using DNA fragment dissected from the entire chromosome 1 (b), 4 (a and c) and 9 (d). (a): FISH without suppression. (b, c and d): FISH with sonicated rice total DNA as competitor. Arrowheads represent chromosome specific signals.

regions correspond to heterochromatin region stained by Giemsa. In human, it has been reported that suppression to non-specific DNA regions is indispensable for painting specific regions (Lengauer *et al.* 1990). Suppression to non-specific DNA regions is also indispensable for painting specific regions in rice. However, painting for specific regions was not detected on the entire chromosome 7 and the acrocentric chromosome for

chromosome 5 using FISH. This may be attributed to the amplified DNA fragments which were almost non-specific DNA consisting of heterochromatin.

Dot blot hybridization

The construction of chromosome region specific DNA libraries was confirmed using dot blot hybridization (Fig. 3). Signals were detected on *XNpb 302* but not on *XNpb 346* and *359*, when RFLP markers *XNpb 302*, *346* and *359* located on chromosome 1 were used for dot blot hybridization. For entire chromosomes 4 and 9, signals were detected at *XNpb 49*, *177* and *247* and at *XNpb 13*, *103* and *293*, respectively. For entire chromosome 7, signals were detected on *XNpb 117* but not on *XNpb 106* and *379*. For the extra chromosome of Acro5a, signals were detected on *XNpb 208*, *292* and *R2232* but not on *XNpb 25* and *297*. These results agreed with our previous observations (Ikeda *et al.*, 1999). Therefore, FISH as well as dot blot hybridization may be utilized for identification of DNA libraries. The results suggested that the chromosome region specific DNA libraries for the acrocentric chromosome could be constructed.

Using RFLP markers not located on target chromosomes, signals were detected on several loci. This suggests that the homology was high between DNA fragments and RFLP markers located on chromosomes except the target chromosome.

Characterization and polymorphism of clones

One hundred sixty and 587 DNA clones from entire chromosomes 1 and 4 were obtained, respectively. Twenty-eight DNA clones in 160 clones and 35 clones in 587 clones

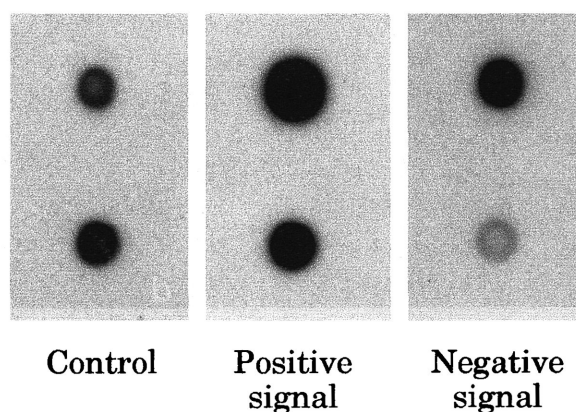


Fig. 3. Southern analysis of rice RFLP clones to PCR products obtained by microdissection. In each autoradiogram, the upper dot is total Nipponbare DNA as a control, and the lower is PCR products of chromosome 7. Positive signal is shown on the left as a control, positive signal using Npb379 on chromosome 7 on the center, negative signal using Npb342 on chromosome 6 on the right.

were characterized (Fig. 4). Twenty-eight clones from entire chromosome 1 have single and multi-copy sequence, respectively (Table 2). Nineteen out of 20 clones showed polymorphism among six varieties. In chromosome 4, 23 clones were single-copy, 16 of these clones showed polymorphism among six varieties (Table 2). Twelve clones were multi-copy sequence. In total, 43 clones (68.3%) were single-copy sequence and 20 clones (31.7%) were multi-copy sequence. Thirty-five clones (55.6%) showed polymorphism among six varieties mainly between Japonica and Indica.

In Japonica variety "Nipponbare", 37.9% of 974 DNA clones indicate multi-copy sequence, 28.8% of 974 clones show polymorphism between Indica Kasalath and Japonica

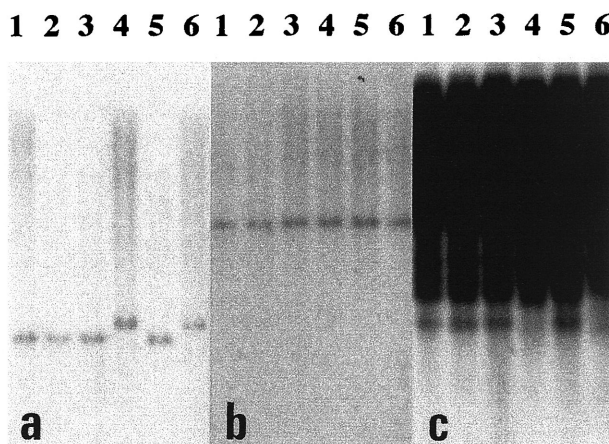


Fig. 4. a-c. Autoradiograms showing characterization of microclones obtained using microdissection in Japonica rice and Indica rice. (a): RFLP (single-copy) using 4W225. DNA was digested with *Bgl* II. Lane 1: Japonica "Nipponbare", lane 2: Japonica "Taichung 65", lane 3: Japonica "Asominori", lane 4: Indica "IR24", lane 5: Japonica "Kinmaze", lane 6: Indica "DV85".

Table 2. Characterization of microclones by Southern analysis

Hybridization signal	Microclones obtained from chromosome specific library of the entire chromosomes			
	Chromosome 1		Chromosome 4	
Single-copy	20	(71)	23	(66)
RFLP	19		16	
No RFLP	1		7	
Multi-copy	8	(29)	12	(34)
Total	28	(100)	35	(100)

(): Percentages relative to total.

Fl 134 (Saito *et al.* 1991). In Indica variety "IR36", 58% and 42% of 210 clones indicate single-copy and multi-copy sequence, respectively (McCouch *et al.* 1988). The frequency for multi-copy in this study is comparable to the result obtained by Saito *et al.* (1991) although the frequency of polymorphism we obtained (55.6%) was higher than theirs. Such differences could be attributed to the type of varieties used in polymorphism survey.

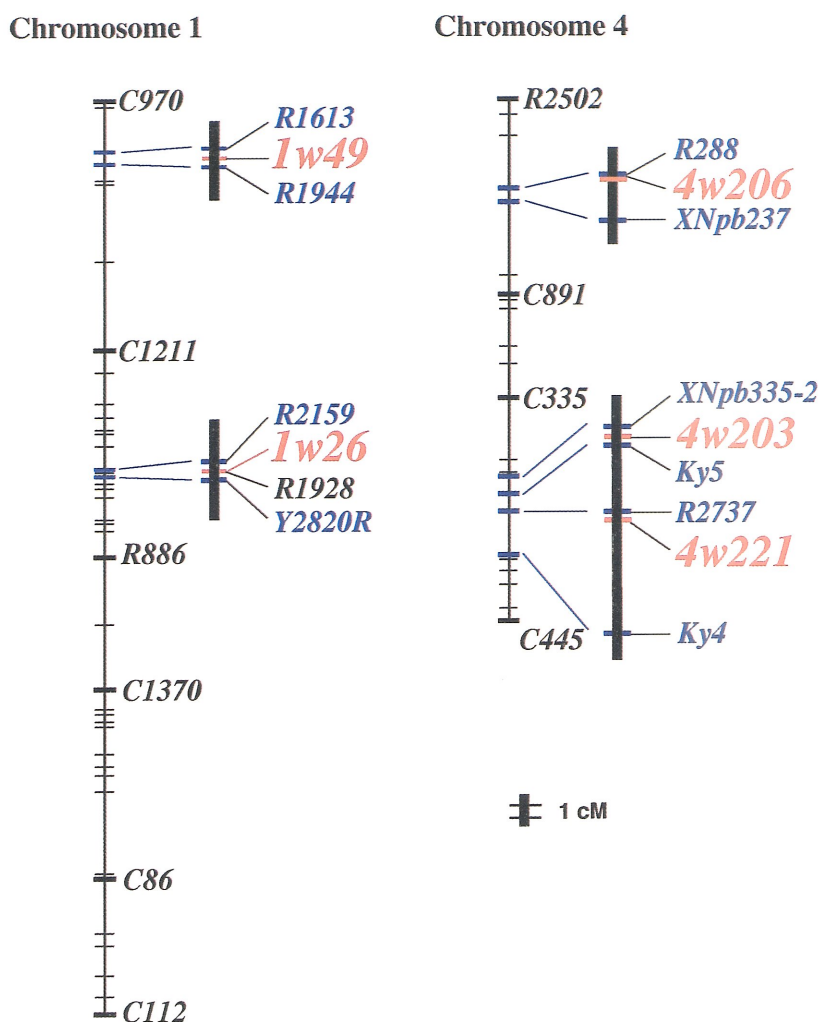


Fig. 5. Location of microclones to RFLP linkage maps (Tsunematsu *et al.* 1996) on chromosomes 1 and 4. Microclones indicated by red numbers were mapped using recombinant inbred lines.

Mapping of clones

On chromosome 1, 1W26 and 1W49 were mapped at the distance of 0 cM from *R1928* and at the distance of 0.8 cM from both *R1944* and *R1613*, respectively (Fig. 5). Our results suggest that 1W26 was located at the region near centromere of the long arm of chromosome 1 whereas 1W49 was located at the short arm of chromosome 1. This supported the previous observations made by Harushima *et al.* (1998).

On chromosome 4, 4W203 was mapped at the distance of 0.8 cM from *XNpb335-2* and 2.4 cM from *Ky5*. The 4W206 locus was mapped at the distance 0 cM from *R288*, whereas 4W221 was mapped at the distance of 0.8 cM from *R2737*. The 4W206 locus was located at the region of centromere; 4W203 and 4W221 were both located at the light stained region on the long arm of chromosome 4 (Ikeda *et al.* 1999).

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