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Z. X. Wang · N. Kurata · S. Saji · Y. Katayose · Y. Minobe

A chromosome 5-specific repetitive DNA sequence in rice (*Oryza sativa* L.)

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Abstract Repetitive DNA sequences in the rice genome comprise more than half of the nuclear DNA. The isolation and characterization of these repetitive DNA sequences should lead to a better understanding of rice chromosome structure and genome organization. We report here the characterization and chromosome localization of a chromosome 5-specific repetitive DNA sequence. This repetitive DNA sequence was estimated to have at least 900 copies. DNA sequence analysis of three genomic clones which contain the repeat unit indicated that the DNA sequences have two sub-repeat units of 37 bp and 19 bp, connected by 30-to 90-bp short sequences with high similarity. RFLP mapping and physical mapping by fluorescence in situ hybridization (FISH) indicated that almost all copies of the repetitive DNA sequence are located in the centromeric heterochromatic region of the long arm of chromosome 5. The strategy for cloning such repetitive DNA sequences and their uses in rice genome research are discussed.

Key words Rice · Repetitive DNA sequence · RFLP mapping · Fluorescence in situ hybridization (FISH) · Physical map

Introduction

Plant genomes contain a large proportion of repetitive DNA sequences (Flavell et al. 1974). Studies of these repetitive DNAs may contribute substantially to our understanding of plant chromosome structure and genome organization (Lapitan 1992). Two basic types of organization have been observed for repetitive genomic DNA sequences. One type of repetitive sequences consist of short repeat units that are tandemly arranged and clustered in specific chromosome regions, such as centromeres, telomeres, and heterochromatin (Bedbrook et al. 1980 b; Peacock et al. 1981; Flavell 1986; Richards and Ausubel 1988; Ganal et al. 1991; Wu et al. 1991; Maluszynska and Helslop-Harrison 1991). The second type of repetitive sequences are interspersed with either unique sequences and/or other different repetitive sequences and are scattered throughout the genome (Bedbrook et al. 1980 a, b; Lapitan 1992).

Recently, a number of studies on repetitive DNA sequences in many higher plants have been reported, such as rye (Bedbrook et al. 1980 b; Appels et al. 1981, 1986), wheat and barley (Dennis et al. 1980), maize (Peacock et al. 1981; Viotti et al. 1985), *Arabidopsis thaliana* (Martinez-Zapater et al. 1986), *Lycopersicon esculentum* (Ganal et al. 1988), and *Oryza sativa* (Wu and Wu 1987, 1992; Zhao et al. 1989; Zhao and Kochert 1992).

The rice nuclear genome contains approximately 50% repetitive DNA sequences, as estimated by Cot analysis (Deshpande and Ranjekar 1980). However, most of these sequences have not yet been identified. We are interested in understanding the chromosome structure and genome organization of rice (*Oryza sativa* L.), with emphasis on cloning and studying the distribution of repetitive DNA sequences from genomic DNA. We report here the isolation and characterization of a chromosome 5-specific repetitive DNA sequence from rice through random genomic DNA cloning, sequencing, RFLP mapping and physical mapping by fluorescence in situ hybridization (FISH).

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Z. X. Wang · N. Kurata (✉) · S. Saji · Y. Katayose · Y. Minobe
Rice Genome Research Program,
National Institute of Agrobiological Resources /STAFF
(Society for Techno-innovation of Agriculture,
Forestry and Fisheries) Institute, 2-1-2 Kannondai,
Tsukuba, Ibaraki 305, Japan

Materials and methods

Plant materials and DNA isolation

A japonica variety 'Nipponbare' and an indica variety 'Kasalath' were used in this study. Genomic DNA was isolated from leaves using the CTAB technique (Rogers and Bendich 1988).

Cloning of rice nuclear DNA

For cloning repetitive DNA sequence, *Hin*dIII-digested fragments of 'Nipponbare' nuclear DNA were separated on 1% agarose gel. DNA fragments in the range of 1.0 to 4.0 kb were eluted out from the gel and ligated to *Hin*dIII-cleaved Bluesript II SK$^+$ (TOYOBO). *E. coli* NM552 competent cells were transformed with the ligated mixture, and ampicillin-resistant white colonies were picked out at random. These clones were screened for repetitive DNA sequences.

Southern blot hybridization

Genomic DNA of 'Nipponbare' and 'Kasalath' were digested with eight kinds of restriction enzymes. Digested DNA samples (2 μg per lane) were electrophoresed in 0.8% agarose gels and transferred onto positively charged membranes (Boehringer). The filters were hybridized with peroxidase-labeled probes in hybridization buffer (Amersham). After overnight hybridization at 42°C, the filters were washed twice with 0.2×SSC/0.1% SDS for 20 min at 42°C. The filters were immersed in the ECL (enhanced chemiluminescence) detection solution (Amersham) for 2 min before they were exposed to X-ray film. One of the clones which hybridized with a large number of genomic DNA fragments was selected and studied as putatively containing a highly repetitive DNA sequence.

Detection of copy number and distribution of the repetitive DNA sequence

The copy number of the repetitive DNA sequence in the rice genome was estimated by using plaque hybridization with a 'Nipponbare' genomic library. 'Nipponbare' DNA was partially digested with *Sau*3AI, and fragments of 5–30 kb were ligated with *Bam*HI-cleaved EMBL3. The library contained on average inserts of 10 kb. The copy number of the repetitive sequence was calculated from the rate of positive plaques to total ones used for hybridization on the basis that one positive plaque should contain at least one copy of the repetitive DNA sequence and the genome size of rice is 4.3×10^8 bp. Several independent positive phage clones were isolated for further analysis of repeat elements.

The distribution of the repetitive DNA sequences in the rice genome was investigated by screening two YAC libraries with the repetitive DNA sequence as probe. The YAC libraries were constructed by Umehara et al. (1995) and consists of 7,000 clones with an average insert of 350 kb, covering the rice genome six times. The distribution of repetitive DNA sequences was determined based on the number of positive YACs obtained in the colony hybridization.

Sequence analysis and repeat unit determination

In order to determine sequences of the repeat units, fragments from the cloned repetitive DNA (G1043) and from two phage clones (λ43-2 and λ43-6) hybridizing with the repetitive sequence were subcloned to pBluescript (SK$^+$, TOYOBO). Several repeat element-containing subclones were used as templates for sequencing using the dye-primer method (Smith et al. 1986). The repeat units were determined by comparing the sequences among the clones using the DNASIS software (HITACHI).

Restriction fragment length polymorphism (RFLP) mapping

A high-density rice RFLP linkage map has been constructed in our laboratory (Kurata et al. 1994) using a F_2 population from a cross of 'Nipponbare' and 'Kasalath'. RFLP mapping was used to locate the repetitive DNA sequence on the genetic linkage map. Southern hybridization analysis using 186 F_2 plants was conducted in the same way as for parent DNA described above. Segregation analysis to determine the map location of this DNA sequence was done according to Kurata et al. (1994).

Chromosome preparation and fluorescence in situ hybridization (FISH)

Rice somatic metaphase chromosomes of 'Nipponbare' were prepared according to the method of Kurata and Omura (1978).

The DNA probe was labeled with biotin-dATP and biotin-dCTP using the random primer labeling system (BRL) according to the standard protocol provided by the manufacturer. Slides with prepared metaphase chromosomes were first treated with 200 μl of 5 μg/ml pepsin at 37°C for 20 min while covered with plastic coverslips, and then incubated with 200 μl of 100 μg/ml RNase at 37°C for 60 min. Chromosomes were denatured with 70% formamide/2×SSC (0.3 *M* Nacl, 0.03 *M* sodium citrate) at 70°C for 5 min. The samples were dehydrated in a series of ethanol solutions and air-dried.

For fluorescence in situ hybridization (FISH), labeled and denatured DNA probe, 40 ng in 25 μl 50% formamide/2×SSC, was applied onto each slide. The slides were covered with glass coverslips and kept in a moist chamber for 48 h at 37°C. The slides were washed with 50% formamide/2×SSC at 42°C for 10 min, and then with 2×SSC at 37°C for 10 min. The slides were put in 1×PBD solution (Oncor) for signal detection.

Signal detection was performed by two cycles of amplification according to the standard protocol provided by the manufacturer (Oncor). DNA was bound with avidin conjugated with fluorescein isothiocyanate (FITC, Oncor) or 5,5′-disulfo-1,1′-di(τ-carbopentynyl)3,3,3′,3′-tetramethylinocarbocyanine (Cy3, Dianova, Hamburg), then with a biotinylated anti-avidin antibody (Oncor) and again with avidin-FITC.

A Biorad confocal laser-beam microscope (MRC600) equipped with appropriate filter sets for red, green, and blue fluorescence was used for chromosome observation. Image recording and processing was performed using the TCL software package (BIORAD) running on a workstation interfaced to the camera. The fluorescence in situ hybridization (FISH) signal for the repetitive DNA sequences was observed in 20 metaphase plates.

Results

Isolation of repetitive DNA sequences in rice

In order to isolate repetitive DNA sequences of rice, we screened randomly cloned genomic fragments. Clones containing repetitive DNA sequences were identified on the basis of their Southern hybridization patterns with nuclear DNA of 'Nipponbare' and 'Kasalath'. Clones showing smear and/or multiband hybridization patterns were picked out as putatively containing repetitive DNA sequences. After a second screening with high stringency, 53 such clones were obtained from about 2,000 random genomic clones. A clone designated G1043 hybridized with genomic DNA and showed a high degree of multi/smear hybridization bands as shown in Fig. 1A. This was one of the highest copy-number clones in the rice genome and was chosen for further characterization.

Detection of copy number and distribution of the repetitive sequence

Hybridization of the G1043 probe to a 'Nipponbare' genomic library (average size of the cloned fragments was 10 kb) showed that about 2.3% of the more than 40,000 plaques screened displayed hybridization signals. This indicated that sequences homologous to the repeat element

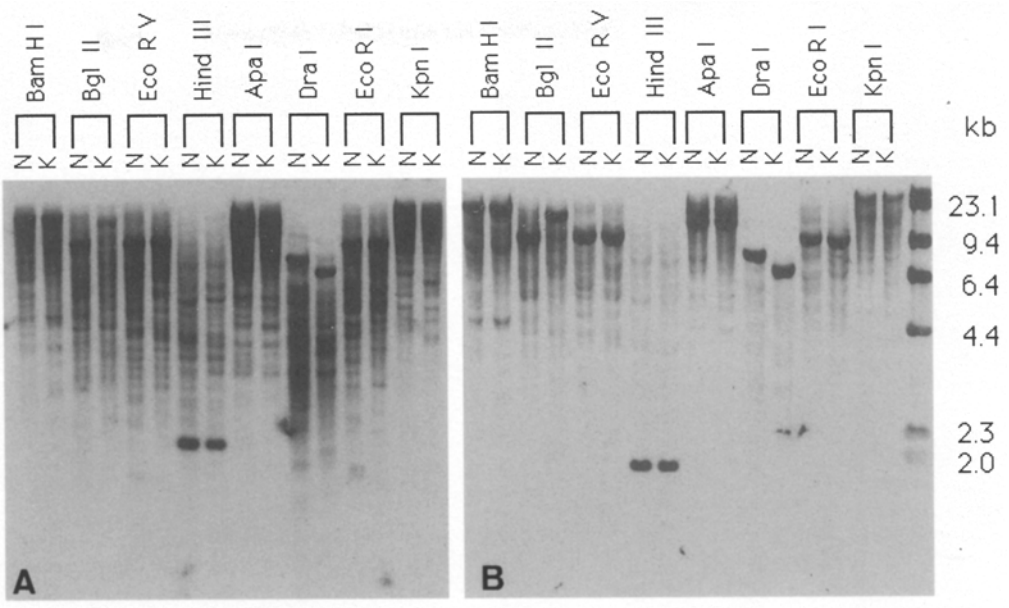


Fig. 1A, B Southern hybridization analysis of 'Nipponbare' and 'Kasalath' genomic DNA with G1043 probe. Genomic DNA (2 μg per lane) of 'Nipponbare' (*N*) and 'Kasalath' (*K*) were digested with *Bam*HI, *Bgl*II, *Eco*RV, *Hin*dIII, *Apa*I, *Dra*I, *Eco*RI, or *Kpn*I. The filters were hybridized to the G1043 (1.7-kb) probe labeled by peroxidase. The hybridization signal was detected by ECL system as described in the Materials and methods. Exposure times were 3 h (**A**) as is the usual case, and 30 min (**B**)

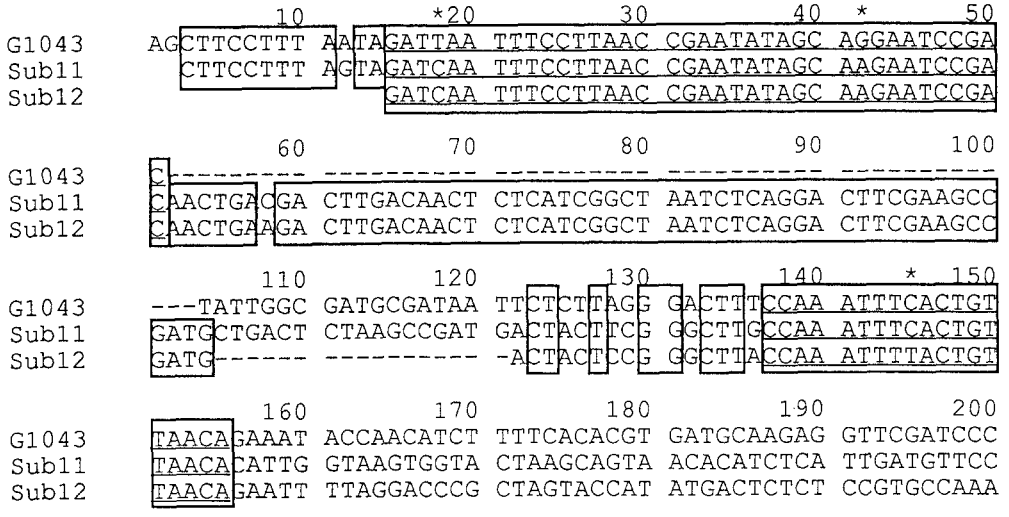


Fig. 2 Comparison of sequences derived from three genomic DNA fragments. Alignment was done manually. *Dashes* indicates gaps introduced to optimize the alignment. Similar sequences between two or three fragments are *boxed.* The same sequences appearing as a single unit are *underlined. Asterisks* indicate the base pair showing single base pair mismatches with the two repeat units among the three clones

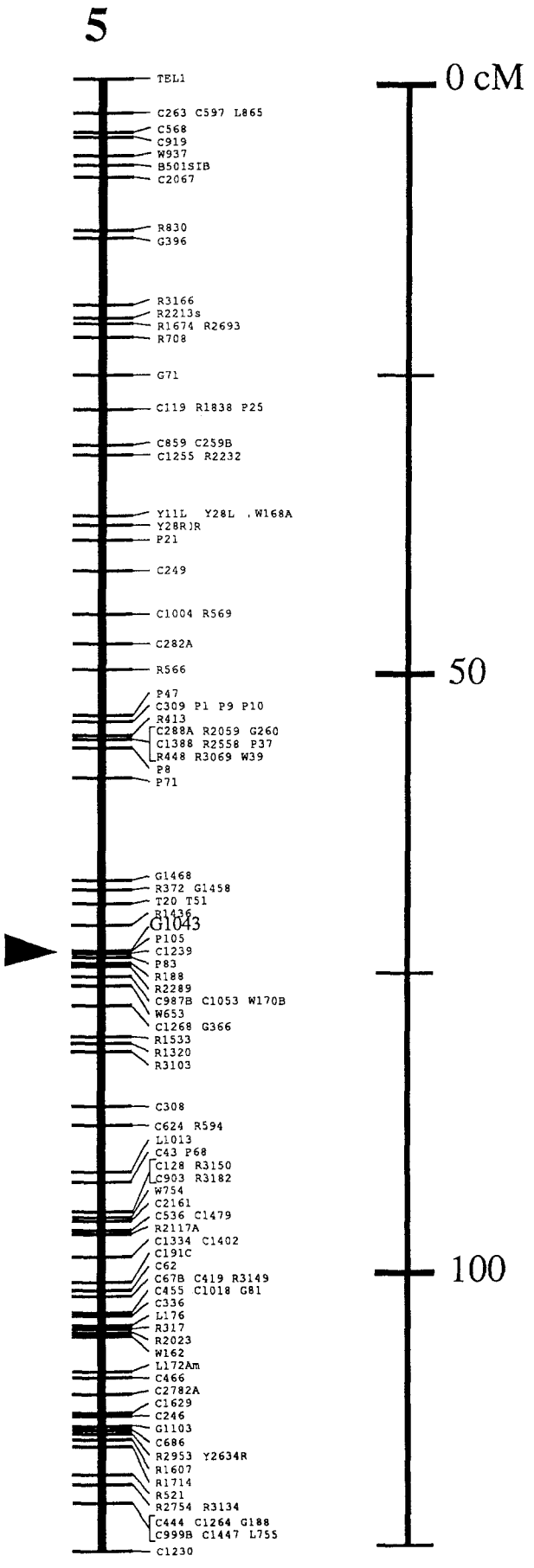
in G1043 are present in at least 900 copies in the rice genome, if we consider the haploid genome size of 'Nipponbare' to be 4.3×10^8 bp.

However, there was considerable variation in the intensity of hybridization signals generated in different plaques, suggesting that individual lambda clones might contain different copy numbers of the repetitive sequences. Therefore, it is likely that the repetitive sequence contained in G1043 is present in more than 900 copies in the rice genome.

Screening YAC libraries with the G1043 probe detected only 7 clearly hybridized YAC clones (corresponding to about 1 clone per one genome coverage), indicating that the repetitive sequence contained in G1043 is concentrated in one or a few specific regions rather than distributed all over the genome.

Detection and sequencing of the repeat element

To determine the length and sequence of the repeat unit contained in clone G1043, 'Nipponbare' genomic clones which showed positive plaque hybridization signals upon probing with G1043 were picked out and analyzed. Hybridization of these clones with G1043 after digestion with each of the 16 enzymes/enzyme-combinations gave single- or multiple-hybridization fragments in each clone of the different digests. Two phage clones, each detected to contain only one single fragment of the repeat element, were used for subcloning and sequencing. The 2 fragments (one a 0.70-kb *Sau*3AI fragment derived from the λ43-2 clone, the other a 0.83-kb *Alu*I fragment derived from the λ43-6) were subcloned as sub11 and sub12, respectively. Sequencing of the 2 subclones, in addition to the clone of G1043



was done twice, producing identical results. Sequences used for the comparison were 600 bp for sub11, 640 bp for sub12 and 1,350 bp for G1043. As shown in Fig. 2, comparison of the three sequences revealed that 37 bp and 19 bp are the common sequences among the 3 clones with only a few base substitutions (asterisked ones) among them. In addition to these core repeat sequences of 37 and 19 bp units, there are a fair number of homologous bases around these core sequences. Thus, it is concluded that the G1043 probe contains at least two 37 and 19 bp sequences as repeat elements. It was also found that the sequences bounded by the two sub-repeat units were different both in length and sequences among the 3 clones.

Location of the repetitive sequence on the genetic map

The location of the repetitive DNA sequence on the rice linkage map was determined by mapping its RFLP fragment. The Southern pattern after a 3-h exposure showed too much smear to do mapping (Fig. 1A). In the case of a 30-min exposure, an intensive unique band revealed in the *Dra*I digest could be used for mapping, as shown in Fig. 1B. By using this intensive RFLP band between 'Nipponbare' and 'Kasalath' (Fig. 1B), we expected a large portion of the repetitive DNA to be mappable. Southern hybridization was conducted with the *Dra*I-digested genomic DNAs of 186 F_2 plants derived from the cross of 'Nipponbare' and 'Kasalath'. Based on the F_2 segregation pattern, this clone could be mapped onto the middle part of linkage group 5 (Fig. 3) of the RFLP map (Kurata et al. 1994). Thus, it was concluded that most of the repeat elements, a part of which is also present in the G1043 clone, are located in the middle of chromosome 5. However, other copies of the repetitive sequences represented by the relatively weak hybridization bands in Fig. 1 remain unmapped because of the complex RFLP patterns.

Physical mapping by FISH

Fluorescence in situ hybridization (FISH) analysis was carried out using somatic metaphase rice chromosomes to determine the chromosome location and distribution of the repetitive DNA sequence. The fluorescence in situ hybridization (FISH) signal for the repetitive DNA copies was detected in about 20 metaphase plates, about 10% of the ones observed. With G1043 as the probe, the hybridization signals could be detected on a specific chromosomal region (Fig. 4A, B). Though we could not carry out clear karyotype analysis for the entire complement, the chromosomes which gave hybridization signals were approximately medium in length and the signal was observed on a distal part of the centromeric heterochromatin of the long arm. No signal was detected on any other chromosomal re-

Fig. 3 Chromosomal location of G1043 DNA on chromosome 5, detected through RFLP mapping and shown by an *arrowhead*

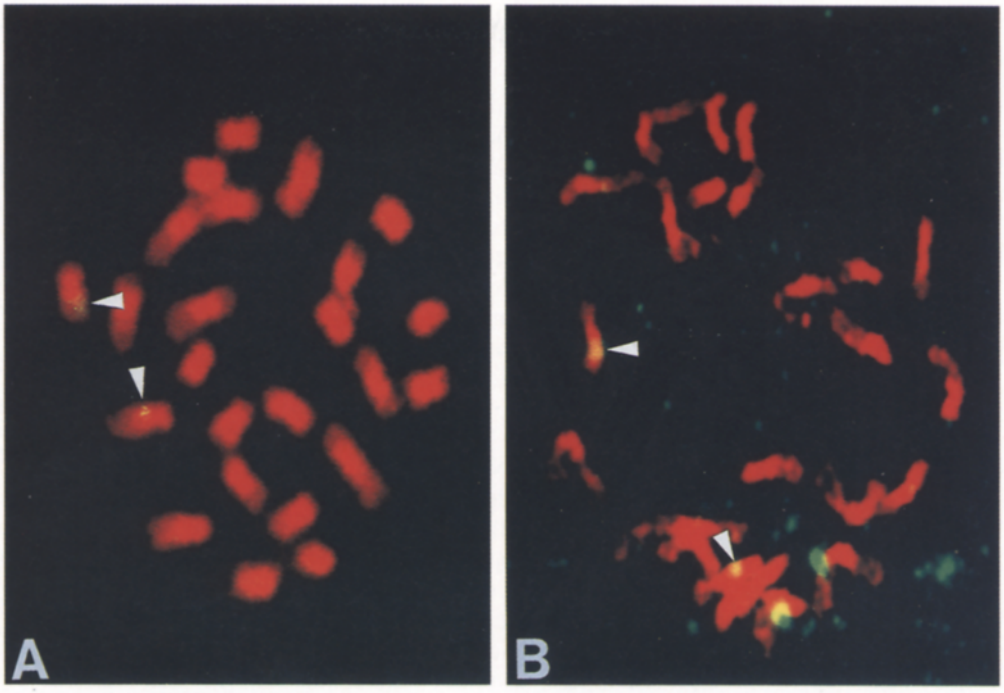


Fig. 4A, B Fluorescence in situ hybridization of rice metaphase chromosomes using G1043 as the probe. *Arrowheads* show the hybridization signals. In both metaphases plates, hybridization signals appear in the centromeric regions of a pair of middle-sized chromosome

gion. Therefore, considering genetic and physical mapping data together, we concluded that most copies of the repetitive sequence are located on chromosome 5 and are concentrated in the centromeric region of the long arm rather than distributed all over the genome.

Discussion

In this report we describe the isolation, characterization, and chromosome localization of a moderately repetitive DNA sequence from rice. Plaque hybridization results suggest that the repetitive DNA sequence is present in at least 900 copies in the rice genome. DNA analysis results showed that the repetitive DNA sequence contains 2 subrepeat units of 37 bp and 19 bp interspersed by other short similar sequences. No homology was found between the sequences cloned in the present study and the repetitive DNA sequences reported in plants or in Genebank (vers. 81), suggesting that this sequence is a new family of repetitive sequences in rice and other plants. Genetic mapping and fluorescence in situ hybridization (FISH) revealed that the repetitive DNA sequences are localized on the centromeric heterochromatin of the long arm of chromosome 5. The absence of any signal detection on any other chromosomal region means that most of the repetitive copies of the sequence are located on chromosome 5. The results from the screening of the YAC libraries with the repetitive sequence also supports this conclusion. If the repetitive sequence was distributed all over the rice genome or on several chromosomes, many YAC clones in the YAC libraries should be positive for the repetitive DNA sequence. That only 1 positive YAC was obtained from every genome equivalent indicated that most of the repetitive copies are included in 1 YAC of some hundred kilo base pairs.

Based on the sequences of the 3 genomic clones, we determined at least two repeat units of 37 bp and 19 bp as the repetitive DNA sequence cloned in the study (Fig. 2). In addition, the two repeat units were interspersed by other sequences also differing in length. The differential length of the interval sequences can be useful for detecting polymorphism for RFLP mapping, as has been demonstrated by Zhao and Kochert (1993).

Chromosome-specific repetitive DNA sequences have been reported in humans for the alpha satellite DNA subfamilies (Choo et al. 1991), and these probes have been used successfully in a variety of studies, such as the detection of polymorphisms (Willard et al. 1986), identification of human chromosomes in somatic hybrids, and detection of aneuploids in interphase cells (Cremer et al. 1986, 1988; Devilee et al. 1988). Similarly, tandemly repetitive sequences, referred to as satellite DNA, have been used for identifying chromosomes in a karyotype (Jones and Flavell 1982; Lapitan et al. 1988; Rayburn and Gill 1987) and for conducting karyotype analysis (Lapitan et al. 1989) in plant species, combined by the technique of in situ hybridization. To our knowledge, our report is the first time a chromosome-specific repetitive sequence has been described in rice, except for rDNA (Fukui et al. 1994). The repetitive DNA sequence should be useful for molecular and genetic studies, for example, the identification of chromosome 5. As is well-known, the somatic chromosomes of rice are very small and similar in size and, consequently, difficult to identify by karyotype analysis. It is easier to

identify chromosome 5 using the repetitive DNA sequence cloned in this study as a probe, combined with the technique of in situ hybridization.

This work started with 2,000 randomly cloned genomic clones with the aim of obtaining high copy-number, repetitive DNA sequences in rice. After a second screening with high stringency, 53 clones were obtained, based on their Southern hybridization patterns, and their copy number was estimated by plaque hybridization. Most of the clones were found to have only several hundred copies, and no clone had more than 2,000 copies in the genome (unpublished data). Therefore, the results of our random survey of the repetitive DNA sequences in rice showed that almost all of them are only moderately abundant, but that there are many kinds of such sequences.

Our strategy for analyzing repetitive DNA sequences involves, (1) cloning genomic DNA at random and picking up the repetitive DNA clones based on their Southern hybridization patterns, (2) cloning other repeat element-containing genomic fragments from the lambda library, (3) sequencing the clones and determining the repeat units, and (4) determining their chromosome location by fluorescence in situ hybridization. It should be possible to isolate chromosome-specific repetitive DNA for each chromosome of rice.

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