

Construction of a DNA library from chromosome 4 of rice (*Oryza sativa*) by microdissection

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ABSTRACT

A simple method to create a chromosome-specific DNA library of rice, including microdissection, amplification, characterization and cloning, is described. Rice chromosome 4 from a metaphase cell has been isolated and amplified by the Linker Adapter PCR (LA-PCR). The PCR products were labeled as probes with DIG-11-dUTP using the random priming method. Southern blot analysis with rice genomic DNA and specific RFLP markers demonstrated that the PCR products were derived from rice chromosome 4. A large library comprising over 100,000 recombinant plasmid microclones from rice chromosome 4 was constructed. Colony hybridization showed that 58% of the clones contained single or low-copy sequences and 42% contained repetitive sequences. The size of inserts generated by PCR ranged from 140bp to 500bp. This method will facilitate cloning of the specific chromosome DNA markers and important genes of rice.

Key words: *Rice chromosome 4, DNA library, microdissection, LA-PCR.*

INTRODUCTION

Rice is not only one of the most important food crops in the world, but also a model plant among the cereals for molecular genetics studies. It has 24 chromosomes and the smallest genome (4×10^8 bp) of any monocot. Some of important genes,

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for example, Ph (phenol staining), Gin-2 (Gall-midge resistance), Xa-1,2 (bacterial blight resistance) are located on chromosome 4 of rice[1]. Most important of all, sequencing of rice chromosome 4 will be carried out by the Rice Genome Project (RGP) in China.

With the development of the Human Genome Project (HGP) in recent years, analysis of restriction fragment length polymorphisms (RFLP) has become a powerful tool for gene tagging, gene manipulation and physical mapping; these methods are also widely used in rice genome research. McCouch et al. constructed the first RFLP map of rice in 1988[2]. Causse et al. enriched their molecular linkage map to 726 markers based on an interspecific backcross population. But there were only 84 markers mapped on chromosome 4[1]. Another linkage map of rice which was derived from a cross between Nipponbare (a japonica variety) and Kasalath (an indica variety) was constructed by Knrata et al. in Japan, and included a total of 1285 RFLP and random amplified polymorphism DNA (RAPD) markers. Using these probes they also constructed a physical map by YAC clones which has been arranged over 52% of the genome length. Their physical map of the rice chromosome 4 contained 114 DNA markers and 258 individual YACs which formed 17 contigs and covered 48% of the chromosome length (about 16.9 Mb)[3, 4]. In China, Hong utilized bacterial artificial chromosomes (BACs) to build a large-scale contig map of rice including 631 contigs which covered 92% of the genome, based on fingerprinting and hybridization[5, 6].

However, most of these markers from RFLP maps in U.S.A. and Japan came from randomly selected genomic clones, cDNA clones and a few markers from other plants, such as oat, barley and maize. These random markers are distributed unevenly along the chromosomes. Many gaps in rice maps still need to be filled. In addition many of these clones are multi-copy probes, and few STS (Sequence-Tagged Site) markers are mapped. Because the genetic distance between two markers doesn't match the physical distance, we need more probes to saturate the map in order to reduce the errors. Cloning by microdissection and microcloning has proved to be an extremely useful technique to obtain more chromosome-specific molecular markers[7, 8]. Thus, our chromosome-specific library of rice can provide a large number of additional chromosomal assigned DNA markers which will facilitate map-based cloning and construction of a high resolution physical map of rice chromosome 4.

MATERIALS AND METHODS

Materials

Oryza sativa L. guangluai NO.4 was kindly provided by Prof. Yang Jinshui (Fudan University). Eight RFLP markers, R2373, R1854, R288, C1100, C1016, C891, C335 and C445 were kindly provided by Prof. Sasaki, the head of Rice Genome Project (RGP) in Japan.

Preparation of metaphase chromosome spreads and microdissection

The procedures for preparation of metaphase cell and isolation of rice chromosome 4 were es-

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as the primers: 5'-ACAGGAAACAGCTATGACCA-3' and 5'-CGTTGTAAAACGACGGCCAG-3'[13].

RESULTS

Microdissection of rice chromosome 4

Rice chromosome 4 was isolated from one metaphase cell (Fig 1) using a micro-manipulator combined with a Nikon microscope and a 1 μ m tip-glass needle. Rice chromosome 4 is easy to identify, as it is submetacentric and the 4th longest chromosome.

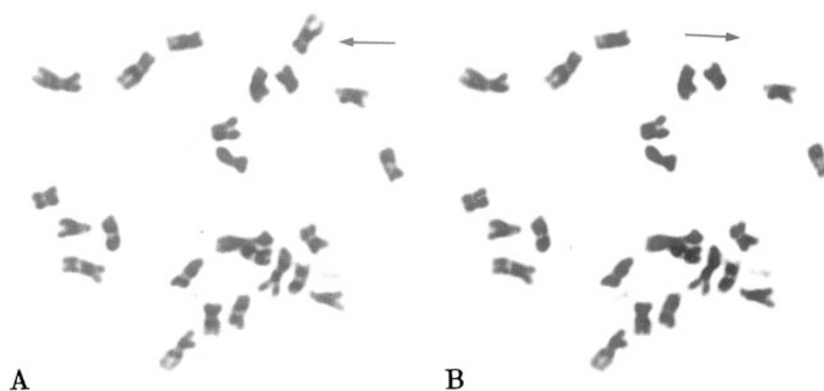


Fig 1. Microdissection of rice chromosome 4.

A. Before microdissection of rice chromosome, arrow indicates chromosome 4.

B. After microdissection of rice chromosome 4.

*Amplification of microdissected chromosome 4 using *Sau 3A I* linker adapter*

From the 1.2% agarose gel electrophoresis pattern (Fig 2), the primary and secondary PCR products were smears of fragments ranging from 250bp-2000bp, and there was no detectable amplification of extraneous DNA in negative control. Appearance of specific bands was not observed, which suggests no preferential amplification of a subset of DNA sequences. When stained with ethidium bromide, the amount of amplified DNA in the secondary reaction is estimated in the μ g range, which ensures enough material for probes.

Characterization of PCR products by Southern blot hybridization

Southern blot analysis showed strong hybridization signals between rice genomic probe DNA and PCR products in lane 3, whereas no signal is seen in the negative control in lane 4; this confirmed that rice DNA sequences have been amplified (Fig 3). Additionally, pooled PCR products were used as probes to hybridize with eight

RFLP markers of rice chromosome 4: R2373, R1854, C1100, C1016, C891, C445, C335, R288. As shown in two recent works[3, 4], these eight DNA markers were selected specially for their even distribution along the linkage map of rice chromosome 4. Among these markers C445 and R2373 were located at the two ends of the linkage map and R2373, R1854, C1100, C1016, C891, R288 were used to construct a rice physical map[3]. Furthermore, C1100, C1016, C891, C445, C335 were selected from a rice cDNA library, and parts of their sequences were released on Gene Bank. So these eight probes are site-specific unique sequences. All of these markers except for R288 demonstrated intensive hybridization signals with amplified products (Fig 4). These results gave further evidence that these PCR products were derived from rice chromosome 4.



Fig 2. Amplified products of LA-PCR
 1: Primary PCR products
 2: Secondary PCR products
 3: λ /Hind III+EcoR I
 4: Negative control

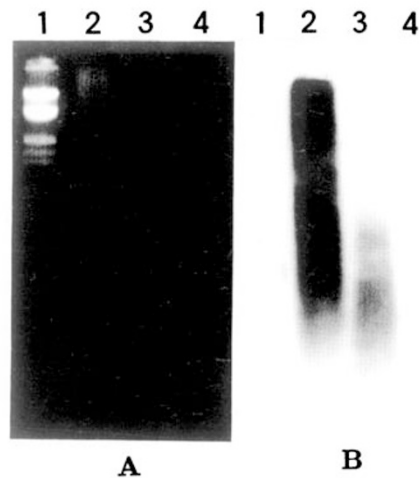


Fig 3. Southern blot hybridization analysis between rice total DNA and PCR products
 A. Before hybridization
 B. After hybridization
 1: λ /Hind III+ EcoR I
 2: Rice genomic DNA
 3: Secondary PCR products
 4: Negative control

Characterization of microclones

Analysis of 500 clones with colony hybridization revealed that about 42% of them

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hybridized either strongly (highly repetitive sequences) or less strongly (moderately repetitive or slightly repetitive sequences) and 58% showed no detectable hybridization (containing unique or very low-copy sequences) (data was not shown). We selected 66 microclones randomly from the recombinant clones to determine their insert size, which ranged from 140 to 500bp with a mean length of 300bp (Fig 5).

Fig 4. Southern hybridization analysis between PCR products and RFLP markers
 A. Before hybridization
 B. After hybridization

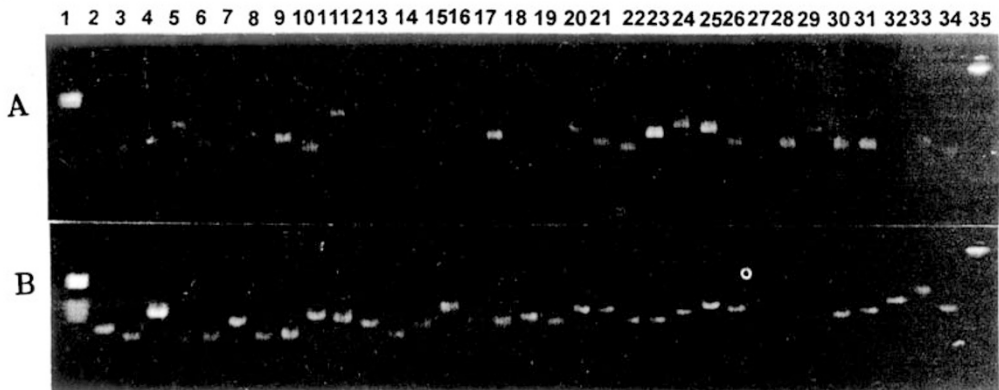
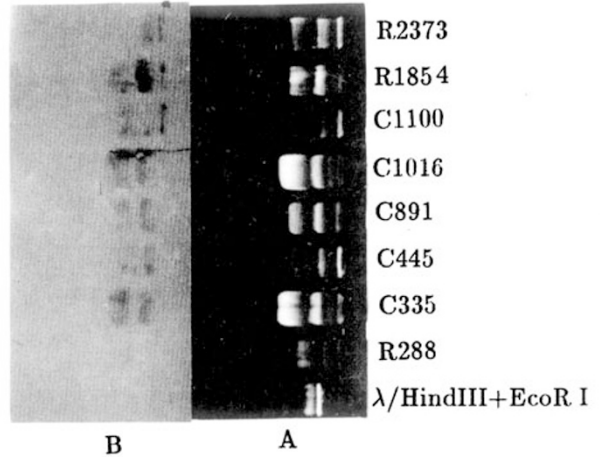


Fig 5. Inserts of recombinant plasmids
 A and B represent different sets of clones
 1: pBR322/Hae III; 2-34: Inserts of microclones; 35: λ /HindIII + EcoR I

DISCUSSION

Because of the lack of special reagents such as human-mouse hybrid cell lines, it is difficult to verify chromosome specificity of PCR products in plant. Southern blot hybridization with total genomic DNA can only prove their origin from certain plants but not accurately from specific chromosomes[14]. Furthermore, some researchers using FISH found that the hybridization signals were dispersed on each chromosome due to repetitive sequences in higher plants[15, 16]. In contrast it is quick and simple to identify where the PCR products come from using specific DNA markers, which have been mapped on designated chromosomes. Cheng et al. succeeded in proving that their PCR products were derived from rice chromosome 5 using STS and microsatellite markers[17]. In order to verify the chromosome-specific origin of our library, firstly Southern blot analysis with genomic DNA was used to prove our products really contained rice sequences. Then, we carried out hybridization with eight RFLP markers that were site-specific single copy probes. The results indicated that our library not only originated from rice chromosome 4 but also covered a fairly high proportion of it. The fluorescence in situ hybridization (FISH) using the secondary PCR products of rice chromosome 4 as probe was also carried out and the same result was gotten as Hernould et al.[15] that hybridization signals were distributed on every chromosome of rice including chromosome 4 (data not shown). Maybe the dispersion of *in situ* hybridization signals was a characterization of higher plants because of high proportion of repetitive sequences. However, this is still a problem deserving of further discussion. Individual and multiple chromosome specific unique probe fluorescent images of high quality by FISH are certainly good means to double check the target chromosome, which we would like to perform in the future.

It is relatively simple and efficient to isolate a large number of microclones containing unique sequences by colony hybridization. 58% of the clones in our library can be expected to contain single or low-copy sequences which is similar to the content of such sequences in the rice genome (50%). This result demonstrates that LA-PCR had no preferences for repetitive sequences. Thus, thousands of microclones with unique sequences can be conveniently isolated and used in screening other rice genomic libraries for the corresponding genes.

Even though RFLP linkage maps of rice have been completed in Japan and America, and the construction of a rice physical map is being carried out, there are still many gaps in the maps. A saturation map of every small genomic region is indispensable for map-based cloning of genes of interest and filling up gaps in a physical map. Currently there are 163,215 ESTs (Expressed Sequence Tags) and about 30,000 STS markers located on human chromosomes[18]. In the rice genome, another 900 RFLP markers were added to the linkage map after the Japanese RGP published their physical map[3]. However, compared with the progress of the Human Genome Project, DNA markers to the rice linkage map have not progressed so far. Thus, it is necessary to construct DNA libraries of each chromosome and to isolate

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large numbers of unique sequence probes from defined chromosomes. The probes from our library can be used to directly create "Sequence-Tagged Sites" (STS) for rice chromosome 4. In addition, the probes can be conveniently used to screen YAC libraries with large inserts. If rice chromosome 4 contains 35.4 Mb, it appears feasible to use unique-sequence microclones to isolate 70-100 corresponding YACs with average insert size of 500kb, to construct contigs for entire chromosome. Our library can provide enough genomic landmarks for high-resolution physical map of the entire rice chromosome 4. The probes can also be used to screen various cDNA libraries for isolating expressed sequences from rice chromosome 4. Most of cDNA sequences have functional significance and can be regarded as candidate genes. Finally, our results will provide a great deal of important molecular information, such as telomere and centromere sequences. These sequences will facilitate locating these sites on physical map and research on the function of plant chromosome.

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