



Preparation of single rice chromosome for construction of a DNA library using a laser microbeam trap

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Abstract

We report the development of a laser micromanipulation system and its application in the isolation of individual rice chromosomes directly from a metaphase cell. Microdissection and flow sorting are two major methods for the isolation of single chromosome. These methods are dependent on the techniques of chromosome spread and chromosome suspension, respectively. In the development of this system, we avoided using chromosome spread and cell suspension was used instead. The cell wall of metaphase rice cell was cut by optical scissors. The released single chromosome was captured by an optical trap and transported to an area without cell debris. The isolated single chromosome was then collected and specific library was constructed by linker adaptor PCR. The average insert size of the library was about 300 bp. Two hundred inserts of chromosome 4 library were sequenced, and 96.5% were aligned to the corresponding sequences of rice chromosome 4. These results suggest the possible application of this method for the preparation of other subcellular structures and for the cloning of single macromolecule through a laser microbeam trap.

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Keywords: Optical scissors; Optical trap; Single rice chromosome; Adaptor PCR

1. Introduction

Rice (*Oryza sativa*) has been used as a model of cereal plants in genetics and molecular biology stud-

ies for two reasons. Firstly, rice serves as staple food for over half of the world's population and is the most important food crop for human. Secondly, the rice genome (430 Mb in size) is the smallest in cereal plants and there are high degrees of synteny among cereal genomes (Gale and Devos, 1998). The International Rice Genome Sequencing Project (IRGSP) launched a project on sequencing the whole rice genome (*O. sativa japonica* Nipponbare). The sequencing strategy involves a clone by clone approach

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; BAC, bacterial artificial chromosome

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bases on the rice genomic map constructed through BAC fingerprinting, end sequencing and hybridizing with chromosome-specific markers (Feng et al., 2002). The construction of a rice chromosome-specific library would be helpful in genome mapping because it will provide chromosomal specific sequences for developing genetic markers. In the present study, optical scissors and optical trap were utilized in the isolation of a single chromosome from a metaphase rice cell. Moreover, the isolated chromosome was used for the construction of a rice chromosome-specific library.

There are two major methods for isolating a single chromosome, namely flow sorting and microdissection. A human chromosome 21-specific yeast artificial chromosome library was prepared by flow sorting (McCormick et al., 1989). This technique can be used to gather a large quantity of specific chromosomes. However, this method depends on the existence of substantial differences both in length and fluorescent pattern between chromosomes. Rice chromosomes are small and 2–5 μm in length in metaphase. The differences in length among rice chromosomes are not prominent, so they are not easy to be enriched by flow sorting. Microdissection is another approach for isolating single chromosome. Mao et al. (1998) constructed a rice chromosome 4 library from a pair of chromosome 4 isolated by mechanical microdissection. A chromosome spread was essential in this method and was technically demanding to perform. Instead of performing a chromosome spread, we endeavored to isolate individual rice chromosomes directly from a cell in suspension.

Optical scissors and optical trap are two kinds of laser micromanipulators. Optical scissors can ablate material for cutting and the optical trap uses radiation pressure to hold microscopic objects (Ashkin et al., 1986). Laser microtechnology is a versatile technique in life sciences, especially in the manipulation of chromosomes. Poneles et al. (1989) reported the cloning of telomere sequences from laser-microdissected polytene chromosomes. Seeger et al. (1991) used laser microbeam to microdissect chromosomes collected by an optical trap and subsequently collected the chromosome segments into a glass capillary. Leitz et al. (1994) focused their research on the laser microbeam trap as an optical tool for manipulating living cells. Optical tools are beneficial in biological research for

two reasons. Firstly, they are sterile and operate in a contact free manner. Secondly, they are controlled by computer, which is easier to perform than mechanical method. The use of optical tools in biotechnology and genome research has been reviewed by Poneles et al. (1994).

We developed a laser micromanipulation system combining the use of the optical scissors and the optical trap (Wang et al., 2004). In this paper, the optical system was used in isolating single chromosome directly from a rice metaphase cell for subsequent cloning. The rice cell was cut open by the optical scissors to release the chromosomes into the suspension. Then a single chromosome was captured by the optical trap, subsequently transferred to a micropipette and aspirated into the micropipette tip. Moreover, the isolated rice chromosome was sonicated and amplified for the construction of a chromosome-specific library by linker adaptor PCR.

2. Materials and methods

2.1. Experimental equipment

The equipment used includes an optical scissors and an optical trap (Fig. 1). A pulsed Nd:YAG laser was used as the light source for the optical scissors. The pulse width of the laser was about 10 ns and the pulse repetition rate was 1 s. The frequency-tripled beam of wavelength 354 nm of the Nd:YAG laser was introduced into the microscope (IX-70 Olympus) through its side port. The pulse energy of the laser can be adjusted by changing optical attenuators in the laser beam path. The power level of each laser pulse was approximately 2 μJ .

A laser-diode was used as the source for the laser trap. Its output power can be adjusted by changing the electrical current of the laser-diode. The maximum output was 60 mW. The near infrared laser output (780 nm) of the laser-diode can hardly be absorbed by water (Berns et al., 1992). This protected the chromosome from damage by the laser beam.

The two beams were amalgamated to one beam on a dichroic mirror (M1), which was transparent for the beam at wavelength 780 nm and reflected the beam at 354 nm. The resultant beam was then reflected by another mirror (M2) onto a 100 \times oil immersion

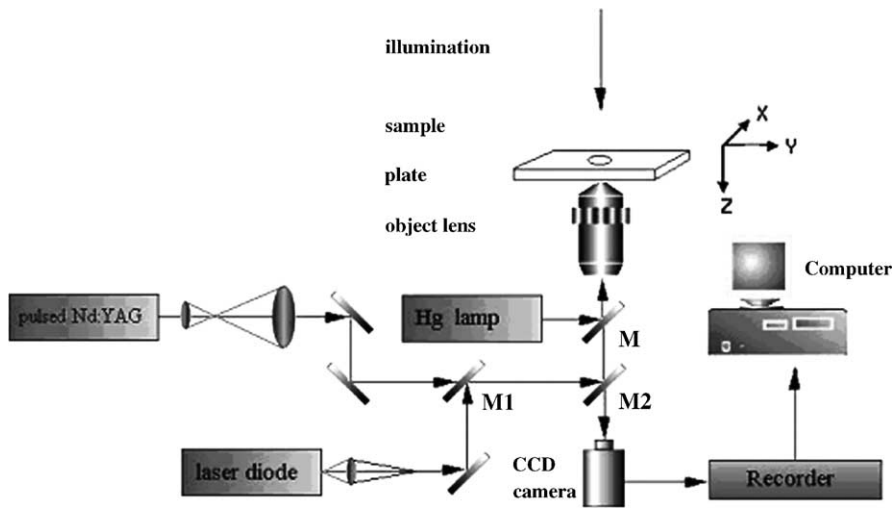


Fig. 1. Schematic diagram of optical scissors and optical trap.

objective (numerical aperture 1.25) and was focused on the sample.

The XY stage of the microscope was motorized and controlled by a computer with a movement accuracy of 1 μm . A video camera was attached to one of the observation ports of the microscope. The whole experimental procedure was monitored on a color screen and recorded by a VCR (Panasonic).

2.2. Preparation of rice root tip cell suspension

Rice (*O. sativa* ssp. *indica* Guangluai 4) mature seeds were germinated and synchronized according to Kurata and Omura's (1978) method. Root tips were digested in 6% (wt. vol.⁻¹) cellulase (Sigma) and 6% (wt. vol.⁻¹) pectolyase (Sigma) for 1 h at 37 °C. The enzymes were then replaced with 0.01 mol l⁻¹ citrate buffer (pH 7.0). The root tips were triturated with a 200 μl tip and left on ice for 8 min. The undigested tissues were settled to the bottom. The upper cell suspension was transferred to a new Eppendorf tube and used for the separation of a single chromosome. All the solutions used were sterile.

2.3. Separation of a single chromosome

We designed a sample chamber by cutting a 5 mm diameter hole in the 1.5 mm thick glass slide and sealed with a 0.18 mm thick cover slip. This sample

chamber was used for the isolation procedures under 100 \times objective. Twenty microliters of cell suspension was added into this chamber and the cell density was adjusted under 10 \times objective. Then a drop of DAPI was added into the cell chamber and the cell chamber was centrifuged for 5 min at 800 \times g to collect the rice cells onto the bottom.

A metaphase cell with clear chromosome shapes was selected as target cell. Turning on the power of the optical scissors, the laser energy was focused on the cell wall, shots were set in succession. After several shots, a broken area was formed in the targeted site. Then, the laser microbeam was focused on another site of the cell wall. After cutting several areas of the cell wall by optical scissors, the cell wall and plasma membrane were broken and the chromosomes were released into the solution. Laser energy and shot number could be adjusted according to the condition of cell lysis.

The optical trap was used to hold the released individual chromosome in focus. The trapped chromosome could then be manipulated in the suspension by the aid of the motorized XY stage. In this way, the optically trapped chromosome was transferred to a clean area with no cell debris and was aspirated into a 2 μm diameter glass micropipette through a IM-300 microinjector (Narishige). The glass micropipettes were fabricated from the autoclaved glass tubing by a PP-830 pulling machine (Narishige). The

isolation procedure from metaphase cell scanning, cell wall cutting by laser beam to chromosome trapping and transferring was controlled by a computer and monitored on the screen.

The micropipette with the chromosome was lifted out of the sample chamber and its outer wall was washed twice with sterile water to avoid cell debris contamination. The micropipette tip with the chromosome was broken into a 0.2 ml Eppendorf tube and stored at -20°C for future use.

2.4. Construction of a single chromosome DNA library

The isolated single rice chromosome was deproteinized by $20\ \mu\text{g ml}^{-1}$ proteinase K (Sigma) at 46°C for 2 h and then cleaved by sonication at 20% energy for 2 s (Bandelin, Sonopuls GM 200). One unit of T₄ DNA polymerase (Rocker) was added for both filling the 3' recessive end and cutting the 3' overhang end to make the sonicated fragments blunt at both ends. Then a synthetic adaptor was added and the mixture was purified to eliminate the protein and salt. The sequence of the adaptor used was:

5'-GAGCGGCCGCTGACTATGTG-3'
3'-CGACTGATACAC-5'

The design of non-adhesive recessive end avoided the formation of adaptor polymer during ligation. Ligation was done at 14°C overnight using Rocker rapid ligation kit. After ligation, the DNA was further purified. Then, the 20-mer oligonucleotide of the adaptor was added as primer to perform the adaptor PCR. The PCR reaction was performed under standard conditions. The process began with seven cycles of PCR in $10\ \mu\text{l}$ reaction mixture followed by increasing the reaction volume to $100\ \mu\text{l}$ and further amplified for 30 cycles. The following cycle condition was used: denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 3 min. Before the first cycle, extension reaction was carried out at 72°C for 10 min to fill the recessed 3' end of the adaptor. After 37 cycles, a final extension at 72°C for 10 min was performed. The amplified DNA fragments were purified and ligated to the pGEM T easy vector (Promega). The recombinant plasmids were transformed into *E. coli* DH 5 α competent cells.

Southern blot hybridization was performed to verify the amplification of the rice DNA. Five microliters of the adaptor PCR product was used as template to amplify for another 30 cycles, then the total PCR product was separated in a 1% agarose gel and blotted onto nylon filter (Hybond N⁺). Rice genomic DNA was sonicated and labeled by random priming. Hybridization was done according to the ECL (Pharmacia) protocol.

The insert length of the library was determined by PCR analysis using the adaptor as primer. The molecular size of the rice DNA insert in each clone was calculated by subtracting the size of the primer sequences (40 bp) from the amplified band.

2.5. Characterization of the single rice chromosome 4 library

The rice chromosome 4 specific library was identified by PCR amplification using rice chromosome 4 specific primers. We designed 18 pairs of primers from 18 specific markers in rice chromosome 4. These primers were used to amplify the chromosome 4 specific sequences from the PCR products from the isolated chromosomes.

Two hundred randomly selected inserts from chromosome 4 specific library were sequenced at one end using the DYEnamicTMET dye terminator kit (Amersham Pharmacia) and analyzed on MegaBACE 1000 sequencer (Amersham Pharmacia). Homology search of the sequences obtained was performed using BLAST program (Altschul et al., 1990, 1997).

3. Results

3.1. Separation of rice single chromosome by optical scissors and optical trap

Sufficient single cells were released into the solution after digestion with cellulase and pectolyase. After centrifuge, the cells were collected at the bottom of the sample chamber and this process had been useful in scanning for metaphase cells with clear shape chromosomes. Fig. 2A shows a rice cell in metaphase. The chromosomes were stained with DAPI and showed bright fluorescence under UV light. The arrows point to the individual chromosomes. There were 24 chromosomes in a rice somatic cell.

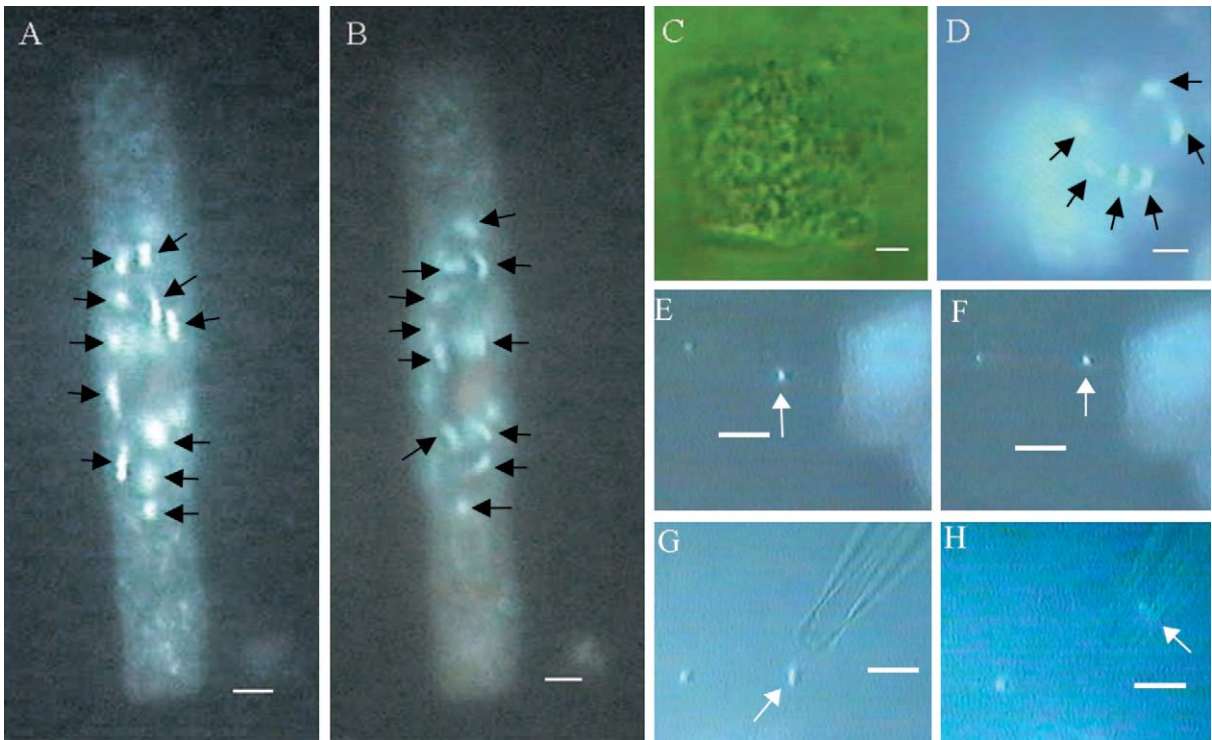


Fig. 2. (A)–(H) The use of optical scissors and optical trap in isolating individual chromosomes from rice metaphase cells. (A) A rice metaphase cell. The chromosomes were stained with DAPI and shown in bright fluorescence under UV light. The arrows point to the individual chromosomes. (B) The same metaphase cell as (A), but was observed in a different focal depth to show the different chromosomes in the same metaphase cell. (C) A rice metaphase cell which was cut open by optical scissors was observed under visible light. The broken cell wall on the right side of the cell was shown. (D) The same cell as (C), but was observed under UV light to show the chromosomes. The arrows indicate the individual chromosomes which were released into the suspension. (E) The use of optical trap to capture and transfer single rice chromosome. The arrow denotes the chromosome which was captured and dragged upwards by the optical trap. A motionless cell and a chromosome (luminescent spot) were shown on the right and left sides, respectively. (F) The same chromosomes as (E), the trapped chromosome had been moved to an upper position as indicated by an arrow. (G) A rice single chromosome was transferred close to a micropipette tip by optical trap. (H) The rice single chromosome was aspirated into the micropipette tip. The arrow denotes the rice chromosome which was already in the micropipette; scale bar was 5 μm .

Different chromosomes can be observed in different focus planes as seen in Fig. 2B. The arrows in Fig. 2B point to the DAPI stained rice chromosomes. If the metaphase cell was scanned in a confocal microscope, then the chromosomes spatial distribution could be clearly demonstrated.

A metaphase cell with clear shaped chromosomes was cut by optical scissors. After the right side of the cell wall was cut open by optical scissors, several chromosomes were released (Fig. 2C and D). Fig. 2C and D showed the same cell while 2C was observed under visible light to show the cell broken state and 2D was observed under fluorescence

to show the released chromosomes from the broken cell.

The released single chromosome was then captured by the optical trap. Further cutting by the optical scissors may be necessary to isolate a particular chromosome free from other chromosomes or cell debris. The trapped chromosome can be manipulated in the suspension with the aid of the motorized XY stage of the microscope (Fig. 2E and F), and transferred to a clean area without cell debris or other chromosomes within the chamber. Then the optical trap was turned off and the target chromosome was aspirated into a 2 μm glass micropipette (Fig. 2G and H). The aspiration accuracy

was about 10 nl. Twenty-seven rice chromosomes were isolated and stored in individual tubes for subsequent adaptor PCR amplification.

Three sizes of micropipettes with tip diameter 1, 2 and 5 μm had been tested for aspiration. We found 2 μm tip diameter micropipette was most appropriate for the collection of individual rice chromosomes.

3.2. Construction of DNA library from a single rice chromosome

The twenty-seven rice single chromosomes isolated were sonicated, ligated individually to a synthetic adaptor and amplified by adaptor PCR. The size of the rice genome was estimated to be 430 Mb, and each chromosome contained about 0.04 pg DNA on average. Considering the limited quantity of DNA template in the isolated chromosome, amplification was first performed in a small volume of 10 μl and enlarged to 100 μl after seven cycles. Thirty-seven cycles PCR reaction were applied in this work in-

stead of the commonly used two round PCR. The amplified product from one individual chromosome can be used to transform 5000 recombinant clones on average.

The adaptor PCR amplification product of the single chromosome yielded smears of fragments when examined by agarose gel electrophoresis (Fig. 3A). We hybridized these PCR products with labeled rice genomic DNA. Rice genomic DNA (lane G) and all the PCR products showed strong hybridization signals, but no positive hybridization band on the marker lane (Fig. 3B, lane M). This clearly indicated that the adaptor PCR products were amplified from rice genomic DNA. The length of the inserts was determined by PCR analysis using the adaptor as primer. The size of the inserts ranged from 100 to 500 bp, with an average length of 300 bp as estimated by agarose gel electrophoresis (Fig. 4). The inserts were smaller than the average length of the fragments in Fig. 3 where the amplified fragments ranged mainly from 250 bp to 2 kb.

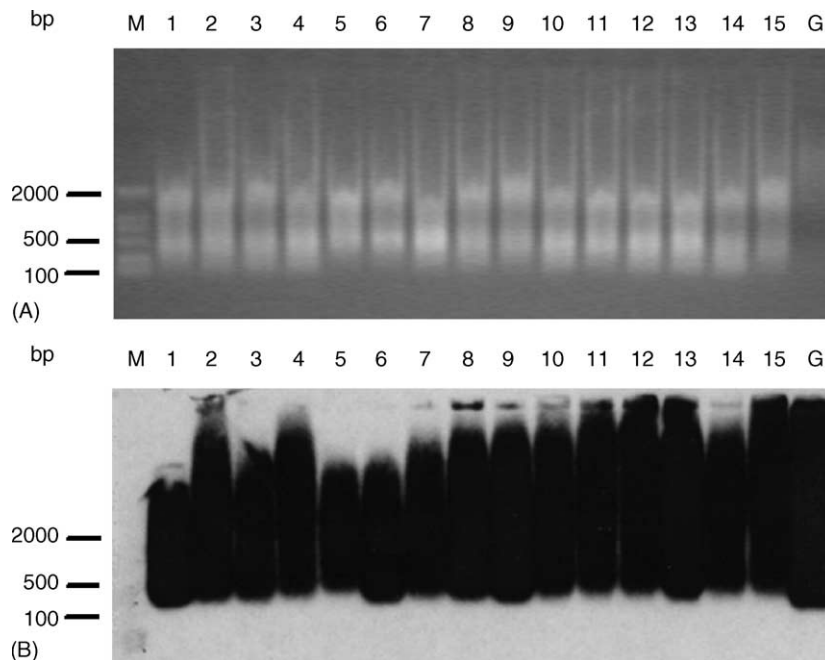


Fig. 3. The PCR products from the individual rice chromosomes were analyzed by agarose gel electrophoresis (A) and Southern blotting analysis with rice genomic DNA (B). Lane M, DL2000 DNA marker (Takara); lane G, rice genomic DNA; lanes 1–15, the adaptor PCR products that were amplified from randomly collected rice individual chromosomes. Rice genomic DNA was sonicated and labeled by random priming.

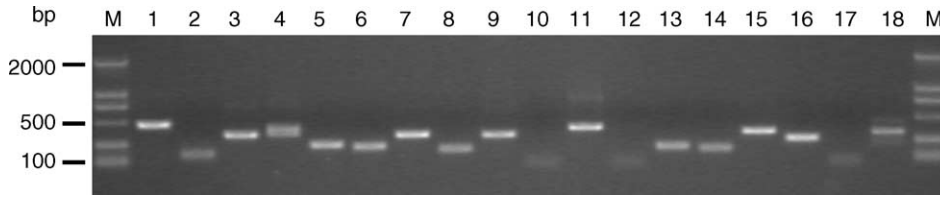


Fig. 4. Determination of the insert sizes in pGEM T easy vector. Lane M, DL2000 DNA marker (Takara); lanes 1–18, individual samples. The molecular sizes of the rice DNA inserts in each clone were calculated by subtracting 40 nucleotides (primer) from the amplified bands.

Table 1
Three pairs of primers designed from three chromosome 4 markers

Marker	Primer sequences	Amplified DNA length (bp)
R10945	5'-CTTCCTTCTGTTTGTGCGAC-3' 5'-TCCTCGTCATCGCTGTACTC-3'	202
C53561	5'-CGGTGGCTGATTACTGCTGC-3' 5'-ATTGTTCCAGTCCAGCCCTC-3'	175
E0025	5'-CCTACTCTACATTGGCCTGG-3' 5'-CAGCAATCTCTCCCCATGTC-3'	175

3.3. Characterization of the DNA library from single rice chromosome

Although the shapes and morphology of rice chromosomes give some clues to their identification, these are not enough to confirm their identity. Therefore molecular characterization should be taken in order to confirm the chromosome origin. We designed 18 pairs of primers of chromosome 4 to amplify the chromosome 4 specific sequences from the 27 single chromosome amplification products. When one single chromosome amplification product was used as PCR template, three right size spe-

cific bands were obtained from primers designed from R10945 (AU071236), C53561 (AU091794) and E0025 (AU093727) (Table 1), respectively (Fig. 5A). These bands were further analyzed by Southern blot hybridization with its corresponding marker sequence and all of them gave positive results (Fig. 5B). Therefore three chromosome 4 specific sequences (R10945, C53561 and E0025) were confirmed in this single chromosome amplification product. So we were able to confirm the identity of the isolated rice chromosome as rice chromosome 4. Similarly, chromosome-specific markers from other chromosomes can be used for the identification of other chromosomes isolated using our method.

This chromosome 4 library was further characterized by sequencing. Two hundred randomly selected recombinant plasmids in the library were sequenced using Sp6 (pGEM T) specific primers. The insert sequences were blast to the sequences of rice chromosome 4 (*japonica*, Nipponbare) (Feng et al., 2002). The whole length of chromosome 4 was about 35.6 Mb, and the centromere was between 9.3 and 9.4 Mb. The inserts were aligned to the chromosome loci where they gave the best identity (from 87 to 98%). One hundred and ninety-three (96.5%) out of 200 inserts made their alignments on chromosome 4.

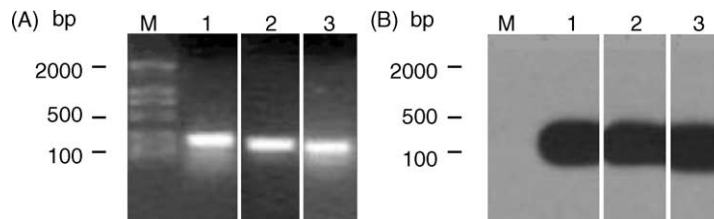


Fig. 5. Identification of chromosome 4. (A) PCR screening of an isolated chromosome using three pairs of chromosome 4 specific primers designed from loci of R10945 (lane 1), C53561 (lane 2) and E0025 (lane 3), respectively. Three unique bands were amplified, respectively, as shown. (B) These bands were further confirmed by Southern blot hybridization with probes of R10945, C53561 and E0025, respectively. Lane M, DL2000 DNA marker.



Fig. 6. The distribution of the cloned DNA fragments on chromosome 4. The entire chromosome 4 was indicated in a dark blue bar. The centromere region was indicated in light blue. The short and long arms were therefore indicated on the left and right sides, respectively. The individual sequences cloned from a single chromosome were indicated in red. Since the entire chromosome was indicated in a small scale, some of the 46 individual sequences were shown at the same positions.

These were distributed among 46 chromosome loci with 13 loci located at the short arm and 33 loci located at the long arm of rice chromosome 4 as illustrated in Fig. 6. The total length of the 200 inserts was 59,458 bp. According to this sequence result, the average insert was calculated to be 297 bp. This was in good accordance with the gel electrophoresis result (Fig. 4). The alignment of the 193 inserts on chromosome 4 covered 11,176 bp. Considering each single chromosome library contained 5000 recombinant plasmids on average, the maximum coverage of this library on chromosome 4 was less than 1%.

Five loci which were 5.72, 26.08, 26.09, 31.15 and 34.43 Mb had more aligned inserts than the others. These sequences were further annotated using gene-prediction software FGENESH to give the possible protein-coding region. All of the coding regions were annotated to be polyproteins. This indicated that many retrotransposons were amplified in our single chromosome PCR product. This was due to the high copy number of retrotransposon that was presented in the rice genome.

Through PCR and hybridization (Fig. 5), three chromosome 4 markers (R10945, C53561 and E0025) were confirmed in the chromosome 4 amplification product. But in sequencing, no other chromosome 4 markers were found among the 200 inserts. This indicated that the ratio of unique sequence was relatively low in our library. Seven inserts (3.5%) out of the 200 inserts showed no homology to rice genomic sequences and also no homology to any known sequences in Genbank. The known sequence of rice chromosome 4 spanned 34.6 Mb and represented 97.3% of the chromosome. So we supposed the sequences with no homology might be rice genomic sequences that had not been identified previously.

4. Discussion

In this report, an optical trap combined with optical scissors was used to prepare isolated rice chromo-

somes directly from a metaphase rice cell. This procedure had several advantages. Firstly, it was easy in sample preparation. The technically demanding procedure of chromosome spread was avoided, and cell suspension was used as experimental material instead. Secondly, chromosome captured by the optical trap provided a cleaner preparation which was free from other chromosomes or cell debris. Finally, the optical micromanipulation system was easier to control than mechanical microdissection.

There were 24 chromosomes in a rice somatic cell. The chromosomes in bright fluorescence showed clear shapes. But it was not effective to isolate all the 24 chromosomes one by one from one single cell. When one cell was exposed under UV light for a longer duration, the chromosome fluorescence would faint, and the chromosome cannot be seen clearly. In our study, five individual chromosomes could be isolated from one metaphase cell.

The 200 inserts from the library established from rice chromosome 4 were blast to rice repeat database in TIGR in order to find the ratio of the repeat sequences in our library. A similarity of >70% was used as a match cutoff. One hundred and sixty-four (82%) inserts were homologous to rice repeat sequences. The repeats were unknown retrotransposon or gypsy retrotransposon elements. In general, there were 50% repetitive sequences in rice, but the repetitive sequences in our library were much higher than 50%. Higher percentage of repeat sequences in our adaptor PCR product might indicate discrimination between repetitive and unique sequences during library construction. In order to exclude the possibility that our adaptor sequence was homologous to rice repeat sequences, we blast our adaptor sequence to rice repeat database in TIGR and no identical sequence was found. This demonstrated that our adaptor sequence had no preference to the repeat sequences in the rice genome.

Repeat sequences in a genome are expected to be cloned at a higher frequency than unique sequences after PCR due to their relative abundance. This explains the high percentage of repeat sequences in our

single chromosome library. Mao constructed a rice chromosome 4 library by microdissection of a pair of chromosome 4 in a metaphase spread and by amplification using adaptor PCR on Sau 3AI digested DNA. It was demonstrated that 58% single or low copy sequences and 42% repeat sequences were presented in their library. Sau 3AI is cytosine methylation sensitive. In plants, methylation islands are clustered in regions of repeat sequences. The methylation islands were not digested by Sau 3AI. Therefore using Sau 3AI digested fragments of rice DNA might give rise to more unique sequences in the constructed library. The difference in the ratio of unique to repeated sequences between the two gene libraries reflects the differences in methodology.

Fig. 5 illustrated the distribution of 200 inserts on chromosome 4. This was based on the assumption that the *indica* and *japonica* genome were highly conserved. Since the individual chromosomes we isolated were from an *indica* variety Guangluai 4, sequence variations may exist. Apart from the seven sequences that were not homologous to rice chromosome 4, the flanking sequences of some homologous sequences also showed differences. Therefore, the accurate map of the chromosome loci was only possible when the whole sequence of Guangluai 4 chromosome 4 was elucidated.

Since the chromosomes remained flexible in suspension during manipulation, there should be less damage in the chromosomal preparation using the optical method than mechanical microdissection. However, the disadvantage encountered in the optical method was the difficulty in identifying chromosomes when they were flexible and curled in suspension. The double beam optical trap that we are developing might be helpful in identifying the chromosomes before separation. The double beam optical trap can hold a chromosome at both ends, in this way; the chromosome is stretched and the chromosome length can be measured (Guck et al., 2000). The chromosome length together with the chromosome stained pattern can help us classify the chromosomes during harvesting and in the molecular identification using specific markers after amplification would confirm the chromosome origin.

The developed method can also be applied to other plant or animal cells. As far as other subcellular structures are concerned, for example, chloroplast, nucle-

oli, etc., this technique is also valuable. The biological material could be cut, trapped and selectively collected. The optical tools are sterile and operate in a contact free manner, so it is particularly suitable for work with sensitive or fragile biological material.

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