High-resolution fluorescence *in situ* hybridization (FISH) for gene mapping and molecular analysis of rice chromosomes

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Fluorescence *in situ* hybridization (FISH) has been an effective technique for physical mapping of genes and repetitive DNA sequences on plant chromosomes. Unique rice genomic DNA sequences ranging from 399 kb (YAC) to 1.29 kb (plasmid) were localized on rice chromosomes using FISH. The detection sensitivity of FISH using rice chromosomes has improved considerably. Extended DNA fibers (EDFs) achieve high spatial resolution and allow quantitative analysis to estimate copy numbers of tandemly repeated sequences in the rice genome. Applications of EDF-FISH and combing techniques have allowed spatial resolutions to increase up to 1 kb between adjacent targets and sensitivity up to 300 bp. The significance of advanced molecular cytogenetic techniques and studies on the rice genome using high-resolution FISH, including EDF-FISH, are discussed.

In this study, we detected genes with several sizes of DNA, including agriculturally important genes. A YAC (yeast artificial chromosome) clone with an insert size of 399 kb was detected at the end of rice chromosome 1 using fluorescence *in situ* hybridization (FISH). A bacterial artificial chromosome (BAC) clone with an insert size of 180 kb was detected at the end of chromosome 2 (Fig. 1A). The BAC clone containing the rice leaf blast resistance gene (*Pi-b*) was revealed at the distal end of the long arm of chromosome 2 (2q2.1) (Figs. 1B, 1C, and 1D). A cosmid (35 kb) with the resistance gene (*Xa21*) against bacterial blight was mapped on the interstitial region of the long arm on chromosome 11 (11q1.3). Detection sensitivity has been increased to detect even a restriction fragment length polymorphism (RFLP) marker of only 1.29 kb. The clone was mapped successfully to the distal region of the long arm of rice chromosome 4 (4q2.1) (Ohmido et al 1998).

These results clearly demonstrated that the physical position of functional rice genes with various sizes can be detected on rice chromosomes. The sensitivity for detecting rice DNAs has been improved 400-fold based on the probe size. DNA sequences that have been physically mapped can be used effectively to fill in gaps in molecular contiguous maps and to determine the actual physical distance between the DNA markers. The structural characteristics of a chromosome where the markers are densely or sparsely distributed can be analyzed by using FISH. The relationship between the gene position and the recombination value can be analyzed.

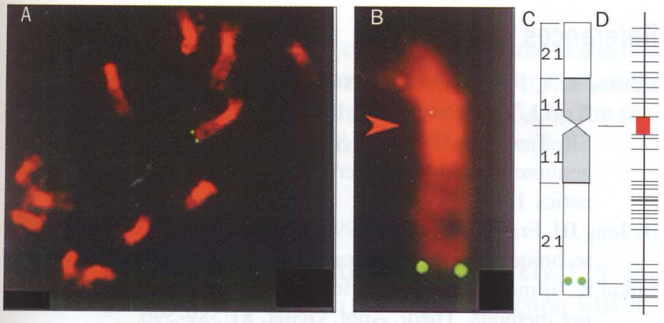

Fig. 1. (A-D) Physical mapping of a BAC clone on rice chromosome 2. (A) The location of BAC clone (180 kb) using FISH. Green fluorescence signals appear at the end of chromosome 2. (B) Enlarged images of the signal-tagged chromosome 2. Centromere position is indicated by arrow. (C) The signal locations are mapped on the rice chromosome as green dots. (D) Position of the clones on the rice genetic map developed by Causse et al (1994). Red box indicates the centromeric regions. A red bar indicates genetic position of the BAC clone. Bar indicates 5 μm.

Highly sensitive physical mapping using extended DNA fibers

In general, the space-resolving power of FISH between highly condensed metaphase chromosomes and interphase nuclei is different. It has been reported that the spatial resolution of two closely located nucleotide sequences by FISH on mitotic chromosomes is 2–5 Mbp and 100 kb on a nucleus. The spatial resolution in pachytene chromosomes ranges from 1.2 Mbp to 120 kb at heterochromatic and euchromatic regions, respectively (de Jong et al 1999).

To analyze the terminal structure of rice chromosomes at the molecular level, FISH was performed using telomere and subtelomeric sequences of rice. In our studies, four different FISH targets such as mitotic chromosomes, somatic nuclei, meiotic chromosomes, and extended DNA fibers were examined. Chromosome FISH revealed the presence of telomere sequences at all the ends of rice chromosomes. Two TrsA loci were also detected in haploid rice plants (Fig. 2A). Identifying rice chromosomes based on the condensation pattern (Fukui and Iijima 1991) before and/or after FISH revealed that the two chromosomes with TrsA were the long arms of chromosomes 6 and 12, respectively. The interphase mapping of TrsA and telomere sequences using diploid plants showed that there were four TrsA sites within a japonica rice nucleus (Fig. 2B). TrsA signals were close to the telomere signals in the interphase nucleus but did not completely overlap.

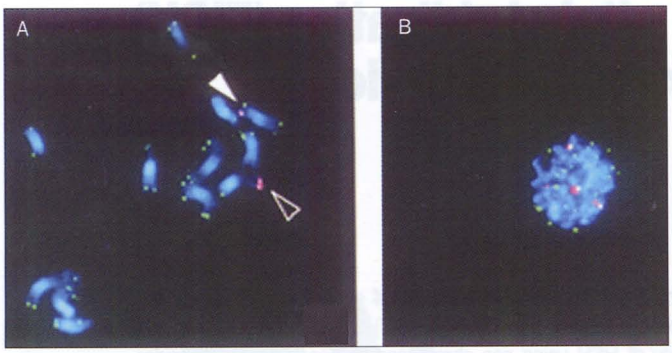

Fig. 2. Multicolor fluorescence *in situ* hybridization (McFISH) on haploid rice chromosomes and nuclei. (A) McFISH for rice prometaphase chromosomes simultaneously using TrsA and telomere sequences as probes. Chromosomes 6 and 12 are indicated by solid and open arrowheads, respectively. (B) McFISH on a rice nucleus. Bar indicates 10 μm.

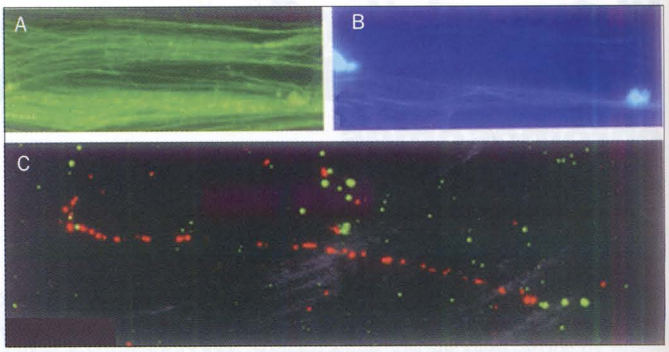

Fig. 3. Visualization of the terminal structure of rice chromosome on extended DNA fibers (EDFs). (A) EDFs stained with YOYO-1. (B) EDFs stained with DAPI. (C) Multicolor FISH on EDFs with TrsA (red) and telomeric sequences $[TTTAGGG]_n$ (green). Bars indicate 5 μm.

Molecular analysis using extended DNA fibers

Partial overlapping showed that TrsA and telomere sequences did not mingle with each other but occupied individual positions. However, when the two DNA sequences were located close together, they could not be resolved using ordinary FISH on chromosomes and nuclei. Thus, the space-resolving power by FISH was improved on DNA fibers released from rice nuclei. Recently, dramatic progress has been made in physical mapping with adjacent DNA clones using FISH on extended DNA fibers (EDFs) in both mammals and plants (Jackson et al 1998, Ohmido et al 2000).

To prepare rice EDFs, rice nuclei were isolated from fresh rice seedlings. The isolated nuclei were then pipetted onto one end of a glass slide and disrupted in a lysis buffer for a few minutes. DNA fibers were stretched by tilting the glass slides to an angle of 45 degrees. When the buffer floated downward to the other end of the slide, DNA fibers were thus released and extended. Each single nucleotide strand was visualized after staining with YOYO-1 (Fig. 3A), a DNA-binding green fluorescent dye that is more intense than DAPI (Fig. 3B).

FISH on the extended DNA fibers using TrsA as the probe depicts clear "beads-on-a-string"-like green fluorescent signal tracks. The shorter stretches of TrsA signals were determined to correspond to the TrsA site from chromosome 6, based on the weaker intensity of the fluorescent signal in the chromosome FISH. Chromosome 12, which had a larger copy number of TrsA than chromosome 6, showed longer stretches of fluorescent signal. Fluorescent patterns of parallel-running linear tracks of red (TrsA) and green spots (telomere) were observed after multicolor EDF-FISH, simultaneously using both TrsA and the telomere sequences as probes (Fig. 3C). Signals from the telomeric sequences appeared as one or a few dots at one end of the TrsA signal tracks, indicating that the telomere sequences were much shorter than TrsA. Results also indicated that the TrsA and the telomere sequences were located in tandem with a few intervening sequences less than a few kilobases long. TrsA and telomere signal tracks were measured using CHIAS III (Kato and Fukui 1998) for the quantitative analysis of EDF-FISH. The copy numbers of TrsA (unit length: 355bp, Ohtsubo et al 1991) on chromosomes 6 and 12 were estimated to be 682 and 231 copies, respectively. For this estimation, the conversion factor, one microscopic length μm equals 3,270 bp nucleotide length, was applied. Both the telomeric repeats on chromosomes 6 and 12 were observed as a few dots of green fluorescence signals and were calculated as 3.2 kb on average. Comparison of the lengths of telomere sequences between indica and japonica rice using EDF-FISH revealed that telomere sequences in indica rice are three times longer than those in japonica rice.

The molecular combing technique is a derivative of the EDF-FISH. DNA combing in conjunction with FISH enables high-resolution visual mapping of the multiple gene clusters on the large DNA fragment (Jackson et al 1999). EDF-FISH with high space-resolving power is now available to quantitatively analyze the length of repetitive sequences. Furthermore, ordering of genes, which is important for chromosome walking and contiguous mapping in genome research, is visually attained by the FISH method.

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Analysis of meiosis in rice after mutagenic treatment

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We studied meiosis in mutagen-treated populations of rice. The treatment consisted of gamma rays (10, 15, and 20 KR), ethylmethane sulfonate (EMS) in 0.2%, 0.4%, and 0.6% concentration for 12 h, and combinations of gamma rays (10, 15, 20 KR) and EMS (0.2%). Cultivar Dubovskiy-129 had normal meiosis with the regular formation of 12 bivalents with the second division of meiosis showing normal anaphases and tetrads. All pollen grains were fertile. The mutagen-treated population showed chlorophyll mutations and various other changes in morphological traits. Meiotic abnormalities were observed in EMS treatments; 26% of the cells showed chromosome fragments. Chromosome stickiness was common in some treatments, including the occurrence of polyploid cells. Variants (20 KR + 0.2% EMS) with univalents were recorded.

In the study of meiosis of cultivars, mutants are an important spectrum of chromosomal variations and changes, and are used to determine morphological traits. Research on meiosis is hampered because chromosomes of rice are extremely small. Also, chromosomes of rice are poorly stained with acetocarmine and aceto-orcein.

In the literature, there is practically no research on meiotic processes in rice. Several authors studied the meiotic behavior of chromosomes in interspecific hybrids of rice and in amphidiploids. However, limited literature is available in Russian on rice cytology.

After mutagenic treatment, a high frequency of chromosomal aberrations, translocations, bridges, fragments, and laggards has been reported.

We studied the meiosis of cultivars and plants of the first generation after treatment by various mutagens to explore the possibility of accelerating breeding work and defining the productivity of rice under conditions of the Almaty region.

Materials and methods

The materials were two cultivars, Dubovskiy-129 and Alakulskiy, one dwarf rice from the world collection VIR (C-5467), three samples of breeding lines (N 348, N 14/282, and N 119/27), and M_1 plants of Dubovskiy-129 after treatment by mutagens. Gamma rays (10, 15, 20 KR), ethylmethane sulfonate (EMS) in 0.2%, 0.4%, and 0.6% concentration for 12 h, and combinations of gamma rays (10, 15, 20 KR) and EMS (0.2%) were used as treatments. Plants were grown in the Main Botanical Garden of the Academy of Sciences RK and in the experimental field (Southern Pribalchashie).

The technique of fixing flowers and staining meiotic chromosomes with some modifications (Khailenko and Sedlovskiy 1998) was followed. Young panicles of rice were fixed at 0600–0800 under 14–15 h of daylight in Almaty and 10–11 h in Southern Pribalchashie. The shoots were fixed with a length of latter internode from 0 to 12 cm and a length of panicle from 2 to 12 cm. The flowers of young rice panicles were fixed on Newcomer. The material was stored in a refrigerator.

Results and discussion

Meiosis was normal for all rice accessions studied. In all meiotic phases, the chromosomes paired completely. The number of bivalents in diakinesis and metaphase I was equal to 12. The meiosis showed normal anaphase and tetrads. The pollen grains were fertile (90–100%).

After mutagen treatment, cv. Dubovskiy-129 in the first generation showed various chlorophyll mutations and numerous deviations in morphological traits. An analysis of meiosis revealed a wide spectrum of chromosome abnormalities in all mutagenic treatments.

The number of cells with fragmentation of chromosomes ranged from 26% (0.2 EMS + 10 KR) to 80% (0.6% EMS). More often, especially after treatment by EMS, besides fragmentation of chromosomes, we observed formation of polyploid (32) cells. Rao (1977) and Sen and Misra (1975) ob-