Variability in rDNA loci in the genus *Oryza* detected through fluorescence in situ hybridization

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Abstract. The 17s–5.8s–25s ribosomal RNA gene (rDNA) loci in *Oryza* spp. were identified by the fluorescence in-situ hybridization (FISH) method. The rDNA loci were located on one-to-three chromosomes (two-to-six sites) within the eight diploid *Oryza* spp. One of the rDNA loci gave the weakest hybridization signal. This locus is reported for the first time in the genus *Oryza*. The chromosomes containing the rDNA loci were determined to be numbers 9, 10 and 11 in descending order of the copy number of rDNA. The application of image analysis methods, after slide preparation treatments (post-treatments), and the use of a thermal cycler, greatly improved the reproducibility of the results. The evolutionary significance of the variability of rDNA loci among the *Oryza* spp. is discussed.

Key words: Fluorescence in-situ hybridization (FISH) – Ribosomal DNA – Genus *Oryza* – Image analysis – NOR variability

Introduction

Rice chromosomes with a large 17s–5.8s–25s ribosomal RNA gene (rDNA) array have been identified as the satellite chromosomes by their characteristics (Fukui and Iijima 1991; Yanagisawa et al. 1991). They are also recognized as the chromosomes with nucleolar organizing regions (NOR chromosomes).

In cultivated rice, *Oryza sativa* L. ssp. *japonica*, one pair of NOR chromosomes was reported by Kurata and Omura (1978) and Fukui and Iijima (1991). This NOR chromosome was designated as no. 11 (Fukui and Iijima 1991) but according to the new system for numbering rice chromosomes, has now been redesignated as no. 9 (Khush and Kinoshita 1991; Fukui and Iijima 1992). By contrast, two pairs of NOR chromosomes were reported in *O. sativa* ssp. *indica* (Wu et al. 1985).

Although the rDNA-containing chromosomes show conspicuous characteristics as satellite chromosomes, they are sometimes difficult to identify morphologically when the copy number of the rDNA units at the locus is small (Leitch and Heslop-Harrison 1992). The in-situ hybridization (ISH) method (Appels et al. 1980; Hutchinson and Miller 1982; Rayburn and Gill 1985) offers a way out of this impasse since it is based on the detection of rDNA loci directly by molecular hybridization. Using this technique one rDNA locus was identified on chromosome 9 in japonica rice (Fukui et al. 1987; Fukui 1990; Iijima et al. 1991) while two rDNA loci were detected in indica rice (Islam-Faridi et al. 1990).

Although ISH is now widely employed in cytogenetic analysis, it is time consuming and strict experimental protocols are needed for its success. Therefore we have developed a reproducible and convenient fluorescence ISH (FISH) technique in conjunction with imaging methods, the use of a thermal cycler, and various post-treatments. As a result, clear fluorescent signals were reproducibly obtained and a new rDNA locus was detected in two diploid wild rice species.

Materials and methods

Plant materials and cytological procedures

Nine rice species, as listed in Table 1, were obtained either from the gene bank of the National Institute of Genetics (Mishima
411, Japan) or Hokuriku National Agricultural Experiment Station (Joetsu 943-01, Japan). Seeds of trisomic lines for chromosomes 9 and 10, and their original variety, IR24, were supplied by Dr. Tsugufumi Ogawa (Kyushu National Agricultural Experiment Station, Chikugo 830, Japan). Seeds were germinated on moist filter paper in Petri dishes at 27 °C under continuous illumination. Root tips 1–2 cm long were excised and fixed in ethanol: acetic acid (1:1). They were stored at −20 °C for about 1 week before examination.

The procedures for sample preparation were according to the protocol described by Fukui and Iijima (1991, 1992) with minor modifications as follows: (1) the glass slides which were used for in-situ hybridization were coated with a 0.1% poly-L-lysine solution (Sigma); (2) enzymatic maceration was carried out in a enzymatic mixture (2% Cellulase Onozuka RS, Yakult Honsha, Co., Ltd., Tokyo, 1.5% Macerozyme R-200, Yakult Honsha, and 0.3% Pectolyase Y-23, Seishin Pharmaceutical Ltd., Tokyo, 1mM EDTA, pH 4.2) in 2 x SSC at 37 °C for 30 min. Secondly, they were treated with 1 mg/ml Proteinase K (Wako Pure Chemical Industries Ltd., Osaka) at 37 °C for 30 min. Thirdly, they were washed in 45% acetic acid for 5 min. They were completely dehydrated through a 70, 95 and 99% ethanol series for 10 min each and were air-dried. Finally, they were treated with 1 mg/ml RNase A (Sigma) in 2 x SSC at 37 °C for 60 min.

The rDNA probe (Sano and Sano 1990) was kindly supplied by Dr. Yoshio Sano, National Institute of Genetics, Mishima 411, Japan. This probe is 3.8 kbp in length and covers most of the coding regions of the ribosomal RNA genes and the flanking spacer regions. The probe was labelled by a random primer labelling method with biothin-dUTP under the supplier's instructions. A 15-μl aliquot of the hybridization mixture containing 100 ng of biotinylated-rDNA in 50% formamide/2 x SSC was dropped on a glass slide. The solution was covered with a cover slip, sealed with liquid Arabian gum and then air-dried. The glass slide with sealed cover slip was placed on a thermal cycler (PHC-3, Techne, Cambridge, UK) that had been remodelled by adding an 80 × 120 mm cast-aluminium flat plate. The programmed heating sequence was 70 °C for 6 min and 37 °C for 18 h.

The cover slips were removed and the slides were washed with 2 x SSC three times and once with 4 x SSC at 37 °C for 10 min each. A 70-μl aliquot of fluorescein isothiocyanate (FITC)-avidin conjugate (0.1 mg/ml, Boehringer Mannheim) was dropped onto the glass slides, which were then incubated at 37 °C for 60 min. After rinsing the FITC-avidin solution with BT buffer (0.1 M sodium hydrogen carbonate, 0.05% Tween-20, pH 8.3) three times at 40 °C for 10 min each, a 70-μl biotinylated anti-avidin solution (1%, Vector Laboratory, Calif., USA) was dropped onto the glass slides which were then incubated at 37 °C for 30 min. After brief washing with BT buffer, a 70-μl fluorescein-avidin solution (1%, Vector Lab.) was applied to each slide. The slides were again incubated at 37 °C for 30 min and then washed thoroughly with BT buffer three times at 40 °C for 10 min each.

Blocking was carried out three times before probe hybridization and before the immunological reaction with 5% bovine serum or goat serum albumin in BT buffer at 37 °C for 5 min.

The slides were counter-stained with a propidium iodide (PI) solution (50 μg/ml in phosphate buffer, pH 6.8, 12.5 mg/ml p-phenylenediamine dihydrochloride, with 90% glycerol) and were then examined by fluorescence microscopy.

Fluorescence microscopy and image analysis

A fluorescence microscope (Axioskop, Zeiss) with B- and G-light excitation filters (B10, G15) was used. A highly-sensitive color CCD camera (HCC-3600P, Floubel, Tokyo) was mounted on the microscope and the fluorescent images were directly frozen in the image frame memories of an image analysis system (VIDAS, Zeiss). All the B- and G-light excitation images were separately recorded in floppy disks and were subjected to image analysis.

Each image has 512 × 512 pixel matrix with 256 steps of a grey value for each pixel as in the images previously analyzed and reported by using the chromosome image analyzing system, CHIAS (Fukui 1985, 1986, 1988, Fukui et al. 1989). Necessary image manipulations consisting of shading correction, normalization, binalization, application of the median filter, and erasing of noise on the binary image, were carried out.

Details of each image filter and image manipulation were as reported previously (Fukui and Kakeda 1990, Fukui and Iijima 1991). Chromosomal areas and the signal regions were separately extracted from the respective G- and B-light images. For the fluorescent signals obtained in the B light, the original grey values were transformed to grey values ranging from 200 to 255. The grey ranges ranging from 0 to 199 were allocated to the pixels of the G-light image that demonstrated mainly chromosome images. The two grey images were combined into a single image.

Pseudocoloration using a look-up-table increased the definition of the image due to the differential coloration generated by computer imaging. The look-up-table was developed by trial and error by repeatedly comparing the original microscopic images with the computer-generated images (Fukui and Ito 1989; Fukui and Kamisugi 1991). The original source images both in B and G light were photographed using reversal color films (Fuji chrome 100, ISO 100, Fuji Photo Film Co., Ltd., Tokyo). Digital images were photographed by a color image recorder (CIR-310 Nippon Avionics, Ltd., Tokyo) using reversal color films (Ektachrome 100, ISO 100, Kodak).

Results

Figure 1a shows the G-light excitation image of the chromosomes of O. sativa, ssp. indica, ev IR36. Two pairs of fluorescent signals were observed in B light (Fig. 1b). Figure 1c shows the integrated image obtained by image manipulation. The current B or G excitation filter used in the experiment visualized either the yellowish-green fluorescence of FITC/fluorescein or the reddish fluorescence of PI. By image processing, the two fluorescent signals were integrated into a single image with yellowish signals on the redh chromosomes. For basic information on the size and number of signals on the chromosomes, the visual recognition of the integrated image was markedly improved by image processing as shown in Fig. 1c. The four signal positions of IR36 were more precisely determined by using the integrated image compared with the two original images.