Development of a quantitative pachytene chromosome map in *Oryza sativa* by imaging methods

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(Received 24 December 2002, accepted 10 April 2003)

A higher GC content region of an *Oryza sativa* chromosome can be specifically visualized by double staining with propidium iodide (PI) and 4, 6-diamidino-2-phenylindole (DAPI). This procedure allows identification of chromosome 9 from the other rice chromosomes at the pachytene stage. Using rice chromosome 9 as a model, an imaging method to construct a pachytene chromosomal map was developed by quantifying the fluorescence profile (FP) of each chromomere. The pachytene map of chromosome 9 consists of twenty-two chromomeres including four chromomeres within the nucleolar organizing region (NOR) and satellite region. The pachytene map was compared with the corresponding somatic prometaphase map and the linkage map. The differences among the three maps indicate that each map depicts specific biological information, which is difficult to be substituted by the other maps.

**Key words:** chromomere, image analysis, *Oryza sativa*, pachytene chromosome, quantitative chromosome map

**INTRODUCTION**

Pachytene chromosomes have higher resolution than somatic chromosomes, due to their elongated structure in meiosis. In pachytene chromosomes, linearly arranged, bead-like, compacted segments named chromomeres are observed. Pachytene chromosome analyses are especially effective in plants with small chromosomes (Fransz et al., 2000). Development of trisomic series in tomato (Rick and Barton, 1954), identification of *Arabidopsis* chromosomes (Ross et al., 1996), and examination of chromosome aberrations in tomato (Havec et al., 1994), etc. were all performed using pachytene chromosomes. In rice, this approach was developed early, and several reports on pachytene chromosomes were published (Shastry et al., 1960; Kurata et al., 1981; Khush et al., 1984) but the results were somewhat divergent. Cheng et al. (2001a) developed a pachytene karyotype, anchored by centromere-specific and chromosomal arm-specific molecular markers.

However, there is no literature report on the quantitative image analyses of the chromomeres. Such an objective analysis method, allowing quantitative image analysis of chromomeres, has long been awaited. Previous development of a comprehensive image analysis system for plant chromosome research (CHIAS; Fukui, 1986; Fukui and Mukai, 1988), and its subsequently improved versions, i.e., CHIAS2 (Nakayama and Fukui, 1997) and CHIAS3 (Kato and Fukui, 1998) lead us to develop a new imaging method, aimed at the analysis of the more complicated structure of the pachytene chromosomes.

In this study, a new image analyzing method for pachytene chromosomes is described. Three different maps, the pachytene chromosome map, the somatic chromosome map and the linkage map of rice chromosome 9 are compared, and the biological significance is discussed.

**MATERIALS AND METHODS**

**Plant materials.** A rice cultivar, Nipponbare (*Oryza sativa* L.) was used throughout the experiment. Anthers, about 0.72–0.75 mm in length, were taken from the young panicles and fixed with a fixative (ethanol 3: acetic acid 1). The root tips were excised, treated with the fixative solution and stored at −20°C until use. For longer storage, the fixative was substituted with 70% ethanol.

**Chromosome preparation.** After rinsing the fixed
anthers in distilled water for about 20 min, pollen mother cells were removed from the anthers, placed on a glass slide and squashed. The slides were frozen on dry-ice, their coverslips removed, and samples were air-dried (Ohmido et al., 2001). Chromosome samples from root tips were prepared according to the enzymatic maceration and air-drying (EMA) method (Fukui and Iijima, 1991; Fukui, 1996b). RNase A treatment (100 µg/ml, 60 min) was performed prior to staining. After 5 min washing with 2× SSC, the slides were dehydrated through an ethanol series (70%, 95% and 99.5%). After air-drying, the samples were stained with a solution containing 1 µg/ml propidium iodide (PI) and 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI). Images of pachytene and somatic prometaphase chromosomes were visualized by fluorescent microscopy (BY60, Olympus) and digitally captured with a CCD camera (PXL1400, Princeton Instrument, USA). Digital images were stored as 12 bit-gray scale images.

Fluorescence in situ hybridization (FISH). The FISH method used was based on a procedure described by Ohmido and Fukui (1997) and Ohmido et al. (1998). The DNA probe, containing a 3.8 kb insert of 17S-5.8S-25S rDNA (45S rDNA, Sano and Sano, 1990), was biotin-labeled by nick translation. The hybridized DNA was detected with avidin-FITC (Vector Laboratories, USA), followed by biotinylated anti-avidin D (Vector Laboratories). The fluorescence of FITC-labeled 45S rDNA probe (Fig. 1h), further confirming that this chromosome region was the NOR. Double staining of somatic prometaphase chromosomes revealed two homologous chromosome 9s with long, stretched, intense PI staining (arrows, Fig. 1b). Two chromosomes, identified as chromosome 9 by their mitotic condensation regions (arrows, Fig. 1e). Two chromosomes, identified as chromosome 9 by their mitotic condensation patterns(Fukui and Iijima, 1991; Iijima et al., 1991; Fukui, 1996), had the NOR at the end of the short arm. Figure 2 shows the averaged fluorescence profiles (FP) along the mid-rib of pachytene chromosome 9 after PI and DAPI staining. The FPs of PI (FP<sub>PI</sub>) and DAPI (FP<sub>DAPI</sub>) staining were similar along whole chromosomes, except for the NOR. Double staining of a certain chromosomal region was observed often attached to the nucleoli (Fig. 1a and 1f). A nucleolus, shaped as a twin ball or ball (Fig. 1d, arrows, Fig. 1g), was observed on most nuclear plates as already reported (Kurata et al., 1981). Thus, most likely, this region was the NOR of chromosome 9, because Japonica rice has one NOR on chromosome 9 in the complement. In addition, partial separation of the homologous chromosome 9s at the pachytene stage was observed (arrows, Fig. 1f). Fluorescent signals (green color) were detected after hybridization with the FITC-labeled 45S rDNA probe (Fig. 1h), further confirming that this chromosome region was the NOR. Double staining of somatic prometaphase chromosomes revealed two homologous chromosome 9s with long, stretched, intense PI staining regions (arrows, Fig. 1e). Two chromosomes, identified as chromosome 9 by their mitotic condensation pattern (Fukui and Iijima, 1991; Iijima et al., 1991; Fukui, 1996), had the NOR at the end of the short arm.

Characteristics of chromosome 9. The ratio of the difference in fluorescent intensity between FP<sub>PI</sub> and FP<sub>DAPI</sub> at the NOR was 5.75, much higher than in other chromosomal regions (0.80–1.28). The fluorescent intensities of DAPI were adjusted to PI for individual chromosomes. We used ten FP<sub>PI</sub> from five chromosome 9s, with little overlapping, and statistically at the same stage in the cell cycle, based on their length (26.27 µm–30.13 µm, 99% confidence intervals) determined by image analysis. The mean length value of the five chromosomes was 27.87 µm (418 pixels). Twenty-two chro-
Pachytene chromosome map of rice

Momeres were visually identified on chromosome 9. The FP$_{PI}$ was used to develop a pachytene chromosome map, because the FP$_{PI}$ and FP$_{DAPI}$ were similar to each other, and because PI stains DNA regardless of its GC content (Fig. 2). The average length of each chromomere was 1.27 µm (19.02 pixels) (Table 1). Differences in staining intensity and in length were observed among chromomeres. For example, chromomere No. 14 was short (15.1 µm) and less condensed. On the other hand, chromomere No. 16 was long (26.0 µm) with large heterochromatic region.

**Image analysis of the pachytene chromosome 9.**

Figure 3 depicts the three major steps of the procedure for mapping of a pachytene chromosome.

1st step: Two chromosome images, of PI (Fig. 3a) and DAPI (Fig. 3b) staining, were stacked and handled as a single image. After noise reduction and normalization of gray levels, midrib-lines were interactively drawn along pachytene chromosome 9 (Fig. 3d). Using the PI and DAPI chromosome images as the references, segmented line-shaped ROIs (“regions of interest”) were interactively drawn at the boundaries between any two chromomeres in these images, and were laid over the 0-gray-level binary image (white) with 255-gray-level (black). Individual chromomeres were numbered, and each chromomere region was pseudo-colored based on its chromomere number (Index image; Fig. 3c). The gray values of FP$_{PI}$ and...
FP<sub>DAPI</sub> along the midrib-line of each homologous chromosome (Figs. 3a, 3b) and the length of each chromomere region of the Index image (Fig. 3c) were obtained.

2nd step: The average of FP<sub>PI</sub> values and the length values of each chromomere were measured using the ten FP<sub>PI</sub> (five chromosomes), and were stored.

3rd step: A bar image of the average chromosome width was developed from the average FP<sub>PI</sub> values, converting black (gray value = 255) to white (gray value = 0) (Fig. 3e: FP<sub>PI</sub> image). The two intermediate images (Figs. 3g, 3h) obtained from the FP<sub>PI</sub> image plus the Chromomere index image (Fig. 3i) lead to the development of the Index idogram (Fig. 3k). Combination of the two intermediate images (Figs. 3g, 3h) with the binary bar image (Fig. 3f) generated a Condensation pattern idiogram (Fig. 3j).

Figure 4 details the process by which the condensed and de-condensed regions of the chromomeres are defined. These regions were initially observed as bright or dark regions on the stained chromosome. The bold line in the graph shows the FP<sub>PI</sub>, whereas the gray line shows the locally averaged values calculated by the moving average. Subtraction of the averaged values from the original FP<sub>PI</sub> values results in an up-and-down line on the X-axis. Each region above the X-axis of the graph corresponds to a condensed or light region. The subtracted gray values of the pixels were replaced with the

### Table 1. Length of each chromomere on chromosome 9.

<table>
<thead>
<tr>
<th>Chromomere (Unit No.)</th>
<th>Length of chromomere (pixels)</th>
<th>Arm symbol</th>
<th>Arm length (µm)</th>
<th>Recombination frequency per mm (cM/µm)</th>
<th>Arm length (µm)</th>
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<td>1 25.0 3.18 sat 5.69 0.00 –</td>
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<td>2 20.6 3.16</td>
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<td>3 16.4 1.07</td>
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<td>4 23.4 2.25</td>
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<td>5 20.7 3.43 p 3.75 0.21 0.52</td>
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<td>6 17.5 3.14</td>
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<td>7 18.1 2.79</td>
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<td>8 22.2 2.91 q 18.40 5.18 2.11</td>
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<td>9 23.3 2.12 (a) (7.39)</td>
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<td>10 16.0 1.34</td>
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<td>11 14.9 1.46</td>
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<td>13 15.5 1.39</td>
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<td>14 15.1 2.96 q</td>
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<td>15 19.6 1.54 (b) (11.01)</td>
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<td>16 26.0 1.92</td>
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<td>17 16.2 2.06</td>
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<td>18 18.8 2.16</td>
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<td>22 19.6 1.77</td>
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<td>Average 19.0 2.26 3.45</td>
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averaged median value of three successive pixels in order to remove the small fluctuation of gray values. As a result, we obtained the standardized data for development of an idiogram with the information of condensation patterns and the position of the chromomeres.

Comparison of three maps: the pachytene chromosome, the somatic prometaphase chromosome and the linkage map of chromosome 9. Figure 5 shows the somatic chromosome map (Fukui and Iijima, 1991; Fukui 1996a), the pachytene chromosome map currently developed, and the linkage map (RGP Public Data, http://web.staff.or.jp/), adjusted for chromosome length. Eight addresses are mapped on the somatic prometaphase chromosome map, and 48 addresses on the pachytene map, with 22 chromomeres. The arm ratio (satellite region excluded) of the prometaphase chromosome 9 is 3.90 (Fukui and Iijima, 1991), whereas that of the pachytene chromosome 9 is 4.92. The arm ratio of the linkage map is 119.13. The overall recombination frequency per unit length (cM/µm) was 3.45 for the pachytene chromosome 9, with values of 0.21 for the short arm and 5.18 for the long arm (Table 1). Centromeric positions are more or less similar between the prometaphase and pachytene chromosomes. However, the position of the centromere on the linkage map (RGP Public Data, http://web.staff.or.jp/) was

Fig. 5. Comparison of the three chromosome maps of rice chromosome 9. From left to right, idiogram for somatic prometaphase stage, idiograms for pachytene chromosomes (Condensation pattern idiogram, Index idiogram), and linkage map.
quite different from those of the two maps. NOR positions were also different, even between prometaphase and pachytene chromosome maps. NOR position in the linkage map can be explained either by lack of its recombination or by lack of detection.

**DISCUSSION**

**Rice chromosome 9.** In this study, structural characteristics of rice pachytene chromosome 9 were defined by image analysis. A study of rice pachytene chromosomes was first reported by Shastry et al. (1960). Kurata et al. (1981) and Khush et al. (1984) also studied rice pachytene chromosomes using Japonica and Indica rice, respectively. In their reports, there was no description of the satellite regions, probably because ordinary staining (such as Giemsa staining of the nucleolus) interfered with the detailed observation of the NOR and the satellite region at the nucleolus. The ideogram currently developed is consistent with that of Kurata et al. (1981) except for the NOR and satellite region. Three chromosomes located within the NOR and satellite region were newly identified by the present study.

DAPI and PI stained the NOR and satellite region differently, as described in our study. PI is a cationic fluorochrome classified as a phenothridium-type dye, which intercalates within the double helix structure of deoxyribonucleic acids. DAPI is a specific intercalator for AT base pairs of DNA. Past and present research indicates that the NOR is a GC-rich region. Using a double staining technique with CMA (Chromomycin A3) and DAPI, Lorite et al. (1997) reported that the NOR had a high GC content. The 45S rDNA, which is the main component of the NOR, shows 60–70% GC content in the coding regions (DNA Data Bank of Japan, http://www.ddbj.nig.ac.jp/). It was also reported that the spacer regions have a GC content of 70.17%, even higher than the coding regions (Takaiwa et al., 1990).

**Image analyses.** Computerized image analysis, to characterize the chromomeres of pachytene chromosomes, has not been available in the past, because the size and fluorescence intensity of chromomeres are quite different from each other. In this study, we established a method to evaluate gray values relative to adjacent regions, to help us discriminate the condensed, chromomeric regions, distributed along the axis of the pachytene chromosomes. The procedure described in this manuscript is based on a method previously developed to extract C-band positive regions in *Crepis* chromosomes, simulating the human visual sense (Fukui and Kamisugi, 1995). The main difference from the previous method is that the current one uses a one-dimensional smoothing method, instead of applying a two-dimensional low-pass filter. The reason for this change is that the result of a low-pass filter treatment of the image was significantly influenced by the gray values of the pixels outside of the chromosomal regions, especially in constricted regions such as the centromeres. The current method was proven to be very effective for the outlining of the condensed regions of the chromomeres. Dawe et al. (1994) and Peterson et al. (1999) also analyzed pachytene chromosomes, using either three-dimensional imaging methods in maize, or FISH methods in tomato, respectively. Our procedure is a alternative approach to pachytene chromosome analysis, which produces quantitative chromosome maps with banding landmarks, similar in aspect to the well-known G-banding of vertebrate chromosomes.

**Advantages of pachytene chromosome mapping.** There are two major advantages in using the quantitative pachytene chromosome map for genetic research. First, in gene mapping, the pachytene chromosome map is more suitable for detailed allocation of genetic information, because more addresses can be assigned on pachytene chromosomes (1 pixel = 67nm) than on prometaphase chromosomes. This also allows a higher resolution gene mapping by FISH on pachytene compared to somatic chromosomes. Cheng et al. (2001b) developed a physical map of rice pachytene chromosome 10, by FISH mapping of bacterial artificial chromosome (BAC) clones. Combining our imaging system with FISH mapping of DNA markers (e.g., genomic anchor clones, cDNAs and transgenic DNAs) would reveal a more accurate physical position of genes and other DNA probes along rice chromosomes.

Second, mapped chromomeres can easily serve as a landmark for precise positioning of structurally unique features, such as heterochromatic and euchromatic regions. Morphological characteristics observed and described in the pachytene chromosomes could not be detected in the corresponding somatic chromosomes. For example, the constriction between the chromomere Nos. 13 and 14 was confirmed only in pachytene chromosomes. The same was true for chromomeres Nos. 16, 17 and 18 which cover the heterochromatic region. These minute morphological characteristics may serve as clues for the analysis of higher chromosomal structures.

On a linkage map, the distance between two loci is measured by recombination frequency, with the assumption that the recombination frequency is even throughout the chromosome. However, there are several reports of uneven recombination frequencies along chromosomes (Fukui and Kakeda, 1990; Gustafson et al., 1990; Künzel et al., 2000). It is likely that, by combining the pachytene chromosome map with the linkage map, better understanding between the recombination frequency and the unit-length of a chromosome can be obtained. This would result in a more accurate, integrated chromosomal map.
We believe that the pachytene chromosome map carries important biological information which is not replaced by the other maps. In combination with other procedures, it could play a crucial role in developing highly integrated chromosomal maps.

The authors thank the members of Chromosome Link for their encouragement. Special thanks go to Dr. Hans J. de Jong, Wageningen University, The Netherlands. Ms. Tomoko Ogihara, National Agricultural Research Center, Hokuriku Research Center, Japan for her technical support in pachytene chromosome preparation, and to Mr. Toshiyuki Wako, National Institute of Agrobiological Sciences, Japan for image analyses on pachytene chromosomes.

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