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Visualization of the ribosomal DNA (45S rDNA) of *Indica* rice with FISH on some phases of cell cycle and extended DNA fibers

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ABSTRACT: The ribosomal DNA (45S rDNA) behaviors during the cell cycle were analyzed on interphase nuclei, prophases, metaphases, pachytene chromosomes and extended DNA fibers in rice (*Oryza,sativa* ssp.*indica* cv.Guangluai No.4) by using high-resolution fluorescent *in situ* hybridization (FISH). The results show that 45S rDNA is located at the ends of short arms of chromosomes 9 and 10. But the signals are much more intense on chromosome 9 than on chromosome 10 in metaphase. Pachytene chromosome has rDNA signal arrays on chromosome 9. Different phases are described and discussed. These results indicate that the activity of rDNA at individual loci may also vary through the cell cycle in rice. On extended DNA fibers, 45S rDNA signals appear as strings of numerous red spots, but some signals are missed in some regions, probably result from weak signals or intergenic spacers.

Introduction

Genome sizes differ greatly in plants due to the presence of different amounts of repeat DNA sequences (Bennetzen, 1996; Graham, 1995). Two basic types of organization have been observed for repeated DNA sequences. One is tandem repeated sequence, and the other is interspersed repeated DNA sequences. Tandem repeat sequences include several types of repeat sequences, such as satellite DNA, ribosomal RNA genes (45S rDNA), 5S ribosomal RNA genes, telomeric repeats (Lapitan, 1992).

The ribosomal DNA repeating unit consists of a transcribed unit (25 S, 18 S and 5.8 S rRNA genes), which is coding regions and highly conserved both in length and in their nucleotide sequences within the plants, and intergenic spacer sequence (IGS) that separates adjacent repeating units, which is highly variable (Long and Dawid, 1980). The length of an rDNA repeat in plant species varies from different species. For instance, the lengths in rice, potato, tomato and wheat are 7.8 kb, 8.8-9.0 kb, 9.1 kb and 18.5kb respectively. Length heterogeneity within individuals was often observed in rice. The length observed for the rDNA repeating unit range from 7.8kb to 18.5kb in plants (Lapitan, 1992). The organization and nucleotide sequences for coding regions and spacers are now available for rice (Takaiwa *et al*., 1990). Numbers of rDNA loci range from 1 to 3 among the species tested in rice (Fukui *et al*., 1994; Shishido *et al*., 2000). But the ribosomal DNA (45S rDNA) behaviors during the cell cycle have not been reported by now.

Fluorescence *in situ* hybridization (FISH) is undoubtedly the most versatile and accurate for ordering

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and positioning specific DNA sequences on the chromosomes (Fransz *et al*., 1996). Pachytene chromosomes are 10 times longer than somatic metaphase chromosomes. Therefore, pachytene FISH also provides a high resolution for mapping DNA probes to specific chromosomal regions. Due to the resolution is increased to 2 kb on extended DNA fibers, the fiber based fluorescence in situ hybridization (Fiber-FISH) technique has been applied for small repetitive sequences (Fransz *et al*., 1996; Jin *et al*., 2004; Ohmido *et al*., 2001), copy numbers (Li *et al*., 2002; Ohmido *et al*., 2000), gap between contigs (Cheng *et al*., 2001), comparative genome research (Jackson *et al*., 2000), transgenic loci analysis, mono-chromosome additional line (Jin *et al*., 2001; Mesbah *et al*., 2000) and the same in plants. This technique has been widely applied and developed yet in plants (Jin *et al*., 2004).

Here, we report the different intensity of 45S rDNA signals in interphase nuclei, metaphases and pachytene chromosomes. We also show the string signals of 45S rDNA on extended DNA fibers in rice (*Oryza,sativa* ssp.*indica* cv.) "Guang Luai 4", which is the representational tested material adopted in China in performing international Rice Genome Program (RGP).

Materials and methods

Plant material

As one of the tested breed in RGP (Rice Genome Program) international consortium, Rice (*Oryza,sativa* ssp.*indica* cv.) "Guang Luai 4" was used for FISH analysis.

DNA Probes and labeling

The plasmid of 45S rDNA cloned in the vector pUC18 (Arumuganathan *et al*., 1994) was kindly provided by Arumuganathan (University of Nebraska, USA). The probe was labeled with digoxigenin with the use of standard nick translation reactions according to the instructions of the manufacturer (Sino-American Biotechnology Company, China).

Preparation of metaphases and pachytene chromosomes

The metaphase chromosomes were prepared according to the published protocol (Song and Gustafson, 1995).

Young panicles with anthers of the rice at various stages of meiosis were harvested and fixed in 100% ethanol:glacial acetic acid (3:1) Carnoy's solution. The suitable anther length for pachytene observation was about 0.7 mm .The anthers were divested from spikelets and kept for maceration in 2% pectinase (SERVA) and 2% cellulase (SERVA) at 28ºC for approximately1.2 hr. After removal of the enzyme solution, the anthers were resuspended in a drop of distilled water, smashed by tip and collected by centrifugation at 2000 g for 5 min. The pellet was resuspended in fresh Carnoy's solution, and mounted onto slides. The slides were dried in a flame.

Preparation of extended DNA fibers

Nuclei were isolated from leaves of 2-week-old rice and the extended DNA fibers were prepared according to Liu and Whittier (1994) and Li *et al*. (2005).

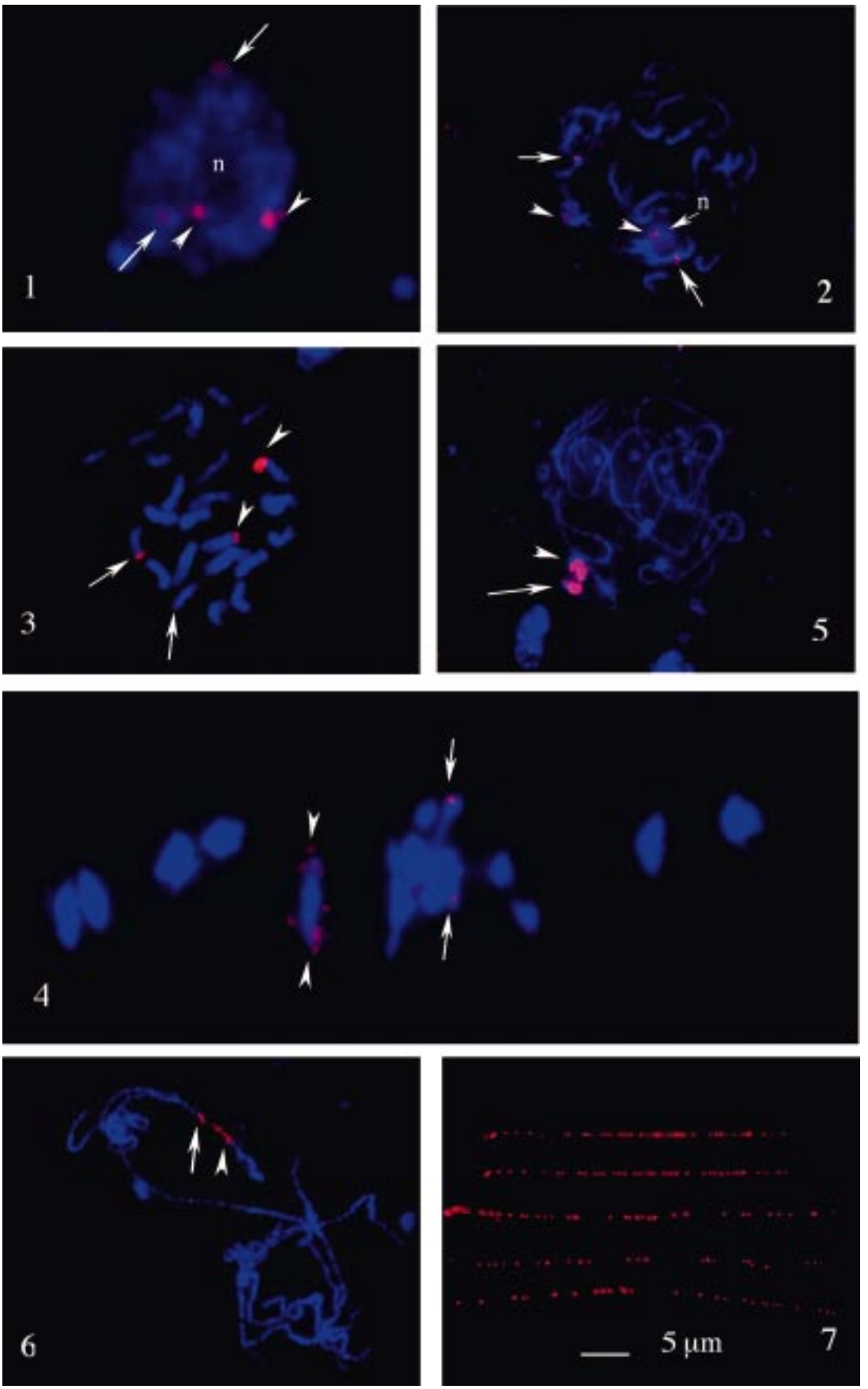
Fluorescence in situ hybridization (FISH)

The FISH of metaphase and pachytene chromosomes was performed according to the published protocol (Jiang *et al*., 1995; Zhong, 1996; Zhong *et al*., 1999), Fiber-FISH protocol was adopted from Jackson *et al*. (1998).

Visualization of hybridized probes

For *in situ* hybridization and fluorescence detection, the slide was incubated with a series of antibodies, mouse anti-dig (Roche), Dig anti-mouse (Roche), and Rhodamine anti-dig (Roche), 37ºC for 30 min for each.

FIGURES. The FISH results of 45S rDNA in rice
The FISH signals of 45S rDNA were revealed on 1. interphase (n: nucleolus)© 2. mitotic prophase, 3. mitotic metaphase, 4. bivalent of meiotic metaphase I, 5. pre-pachytene chromosomes, 6. late-pachytene chromosomes, 7. extended DNA fiber of *Indica* rice. (Arrowheads indicated the intense signals and arrows indicated the weak signals.)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The slide was then washed with PBS for 3∞5 min, and counterstained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI). Chromosomes were observed with an Olympus BX60 fluorescence microscope equipped with Sensys 1401E CCD camera. Red, green, and blue images were captured in black and white with different filters. The images were merged and pesudocolored in the computer using software V^{++}.

Results

Figure 1 shows that there are four signals of rDNA in *Indica* rice interphase, indicating that *Indica* rice has two-rDNA locus. Two of the signals are obviously stronger than the other two, implying that the two-rDNA locus has different copies in *Indica* rice. In late prophase the nucleolus begins dissolving, but some remnants of the nucleolus are still visible (n and arrows in Fig. 2) and one rDNA locus is located in the nucleolus (n and arrows in Fig. 2).

In metaphase the rDNA loci are located at the ends of short arms of chromosomes 9 (arrowheads in Fig. 3) and 10 (arrows in Fig. 3), consistent with the previous study (Fukui *et al*., 1994; Gong *et al*., 2002).The rice chromosome 9 and 10 were identified by their unique characters, such as chromosome 9 is the second smallest chromosome in rice, and chromosome 10 contains considerable heterochromatin on the entire short arm, which were described by Fukui and Gong (Fukui *et al*., 1994; Gong *et al*., 2002). They were also discriminated from arm ratio. It is obvious that the intensity of the rDNA signals is quite different on metaphase chromosomes; the signals of rDNA on chromosome 9 are more intense (arrowheads in Fig. 3) than those on chromosome 10 (arrows in Fig. 3). In metaphase I of meiosis, the signal of rDNA on one bivalent is more intense than that on the other (Fig. 4). It is very interesting that the signals on pachytene chromosomes are contracted in pre-pachytene (Fig. 5) and appear as array beads, 3 to 6 signals are arranged clearly in late-pachytene (Fig. 6), like the signals detected with fiber-FISH (Fig. 7). During prophase of meiosis, the condensation of rDNA sequences is dynamically changed. For further study of the organization of rDNA, Fiber-FISH was applied. On extended DNA fibers, 45S rDNA signals appear as strings of numerous red spots, which are in typical "beads-on-a-string" pattern (Fig. 7), and the signal string is so long that we coul not measure the undoubted length. But some signals are missed in some regions, probably due to weak signals or intergenic spacers.

Discussion

It is certain that numerous rRNA genes sustain ribosome biosynthesis in species. These rDNA sequences with high copy numbers include active and inactive rRNA genes, which result from condensation and decondensation of the chromatin. In wheat, both decondensed and condensed 18S-5.8S-26S rDNAs have been observed in the nucleolus while only condensed rDNA observed outside the nucleolus (Leitch *et al*., 1992). Decondensation of the ribosomal gene chromatin during the cell cycle proceeds in different ways, depending on cell type and species (Leitch, 2000). In previous study, Naganowska and Zielinska (2004) have analyzed rDNA loci in the *Lupinus* genome during the cell cycle, and observed two clearly distinct sites in the interphase, often in the distant regions of the nucleus. Outside the large nucleolus is a darker area. They claimed that these were probably clusters of compact chromatin called perinucleolar knobs (Jordan, 1984) comprising inactive rDNA. Montijn *et al.* (1998) assumed that these knobs might represent the distal and the proximal regions of an active NOR in a study of the nucleolar organizing regions during the cell cycle in two varieties of *Petunia hybrida*. In our study, we also found two intense sites in the interphase, which were often located in the dark blue regions, presumably represent the prenucleolar bodies with condensed chromatin and high copies. The array beads signals on late-pachytene chromosomes (Fig. 6) suggest that one or both NORs of chromosome 9 become active during a relatively short period. Therefore, we think the activity of rDNA at individual loci could also vary through the cell cycle in rice. Iwano *et al.* (2003) indicated that rDNA is transcribed dynamically in a time- and region-specific manner over the course of the cell cycle in barley by analysis of rDNA localization using *in situ* hybridization (ISH) under scanning electron microscopy (SEM).

Length heterogeneity has been found within and among species as well as within individuals in rice by surveying spaces-length variation in rDNA in two cultivated rice species and their wild relatives (Sano and Sano, 1990). In present study, different intensities of signals of two-rDNA locus indicate that two kinds of rDNA with different copy numbers and lengths exist in rice. Different species contain different lengths of rDNA repeating unit because of rapid divergence of the intergenic spacer DNA. For example, the IGS length of moss *Funaria hygrometrica*was is 5150 bp, and the fission yeast *S.pombe* IGS length is 3.45 kb, versus 2.5 kb for *S.cerevisiae* (Liu *et al*., 1997). The IGS length of

rice rDNA genes is 2200bp (van den Engh *et al*., 1992), versus 3200 bp for potato (Heng and Tsui, 1998), 29.7 kb for human (Bassy *et al*., 2000). The two species of *Chlorophytum*, namely *C. borivillianum* and *C. comosum*, both with 2n = 28, are also reveal diversity in copy numbers and localizations of rDNA sites (Lavania *et al*., 2005). An important aspect in the evolution of rDNA is that the origin of new loci and a number of mechanisms has been proposed (Reed and Phillips, 2000). Mechanisms include: (1) chromosomal rearrangements (translocation and inversions of chromosome arms); (2) amplification of 'orphon'-like cistrons (Childs *et al*., 1981); (3) reinsertion errors during cistron amplification; and (4) mobile genetic elements closely linked to rDNA that move rDNA arrays during transposition (Syvanen, 1984). Movement or rDNA loci among chromosomes in patterns similar to mobile genetic elements was demonstrated in species of *Allium* (Schubert, 1984; Schubert and Wobus, 1985). Regardless of the mechanisms of rDNA transfer to a new place on the chromosomes, new rDNA clusters must be stabilized. This stabilization can be achieved by selfing or crossing with similar genotypes and by non-Mendelian epigenetic mechanisms of *En_Spm*-mediated intragenomic transfer of rDNA (Raskina *et al*., 2004).

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