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Physical Mapping of 5S and 18S-26S Ribosomal RNA Gene Families in *Allium victorialis* var. *platyphyllum*

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A physical map of the 5S and 18S-26S rRNA genes was determined using bi-color fluorescence *in situ* hybridization technique in *A. victorialis* var. *platyphyllum*. 5S rRNA genes were positioned in the intercalary regions of the short arms in homologous chromosomes 6. Two major loci of the 18S-26S rRNA genes were detected in the secondary constrictions flanking with a pair of satellite and terminal region of short arm in chromosome 4. And two additional minor loci were heterotype, representing one signal on the terminal region of the short arm in one homolog of chromsome 2, and other on one homolog of chromosome 6 with linked 5S rRNA loci. In addition chromomycin A_3 (CMA_3) fluorescent banding method was used to identify the relation between Nucleolus Organizer Region (NOR) sites and CMA_3 positive heterochromatin sites. In homologous chromosome 4 showing 18S-26S rDNA hybridization signals revealed also distinct CMA_3 positive band.

Keywords: physical map, *A. victorialis* var. *platyphyllum*, bi-color fluorescence *in situ* hybridization, CMA_3 fluorescent banding

In situ hybridization (ISH) technique has been used to detect the chromosomal location of specific genes or DNA sequences directly on chromosomes in cytological preparations. Fluorescence *in situ* hybridization (FISH) techniques using fluorochromes allow the visualization of multicopy gene families, such as the 5S and 18S-26S ribosomal RNA genes (rDNA) on plant chromosomes. The importance of the rDNA cluster is illustrated by the high degree of conservation of sequences, the larger number of gene copies, and the localization of these genes at specialized chromosome regions (Delany *et al.*, 1994). Both the 18S-5.8S-26S and 5S rDNA are present as multigene families organized in long tandem arrays and their distribution and expression has been studied in detail in several plant species (Bergey *et al.*, 1989; Skorupska *et al.*, 1989; Mukai *et al.*, 1990, 1991; Griffor *et al.*, 1991; Lapitan *et al.*, 1991; Ricroch *et al.*, 1992). In yeast, the 5S and 18S-26S ribosomal RNA genes are in juxtaposition in the same locus, whereas in higher eukaryotes they are organized in separate loci (Appels and Honeycutt, 1986). Several new loci of 5S and 18S-26S rRNA genes in wheat and barley were detected by ISH or FISH analysis (Mukai *et al.*, 1990, 1991; Leitch and Heslop-Harrison, 1992; Jiang and Gill, 1994). Many authors concluded that the phylogenetic relationships based on 18S-26S and 5S rDNA loci agreed with other types of data regarding divergence of repeated DNA and chloroplast DNA of diploid *Aegilops* species (Dvořák and Dubcovsky, 1996; Mason-Gramer and Kellog, 1996; Tsunewaki, 1996).

In *Allium sativum*, the 18S-26S rRNA gene families are located in the Nucleolus Organizer Regions (NORs) (Flavell and Smith, 1974; Flavell and O'Dell, 1979; Appels *et al.*, 1980). Besides the major NORs, additional minor loci of NOR had been reported by FISH using 18S-26S rDNA (Hizume *et al.*, 1995). Therefore physical map using FISH was more rewarding than cytogenetic map using Ag-NOR banding method. Moreover the chromosomal location of NORs has been studied by means of chromomycin A_3 (CMA_3) staining and fluorescence *in situ* hybridization in *Picea* (Hizume and Kuzukawa, 1995), *Cicer* (Galasso *et al.*, 1996) and *Medicago* (Calderini *et*

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al., 1996).

In the present work, we describe the results obtained from FISH revealing the precise chromosomal locations of the rDNA in *A. victorialis*. Hybridization patterns of the two ribosomal RNA gene families can be used to identify homologous chromosomes and to provide additional information on the chromosomal rearrangement from minor loci of 18S-26S rDNA.

MATERIALS AND METHODS

Plant materials and cytological preparations

Allium victorialis var. *platyphyllum* was collected from natural populations of Ulreung Island, Korea. Chromosomal preparations for CMA_3 banding and FISH experiments were made as described by Mukai *et al.* (1990).

CMA_3 banding and Fluorescence *in situ* hybridization (FISH)

Chromomycin A_3 (CMA_3) fluorescent staining method carried out by the modified procedures of Hizume *et al.* (1989). The slides were placed in McIlvaine buffer, pH 7.0 for 30 min and were treated with 0.2 mg/ml distamycin A in McIlvaine buffer for 10 min. The slides were rinsed in the buffer containing 5 mM $MgSO_4$ for 10 min after the coverslips were removed in McIlvaine buffer. The slides were stained with 0.1 mg/ml CMA_3 for 10 min, and rinsed in the buffer containing 5 mM $MgSO_4$ for 10 min. Finally, the slides were mounted with non-fluorescent glycerol and incubated at 4°C overnight to stabilize the fluorochrome. CMA_3-bands were observed under an 450 nm Zeiss microscope with B-filter cassette. Photographs were taken with Kodak EKTAR 1000.

Plasmids, pTa794 (Gerlach and Dyer, 1980) and pTa71 (Gerlach and Bedbrook, 1979) used as the probes for 5S and 18S-26S rDNA of wheat respectively. The pTa794 had the 120-bp 5S rDNA region plus a 290-bp spacer. Plasmid pTa71 had one unit of 18S-26S rDNA gene (8.9 kb). The 5S and 18S-26S rDNA were labeled with digoxigenin-11-dUTP (Boehringer Mannheim) and biotin-16-dUTP (Boehringer Mannheim) respectively by means of nick-translation (Gibco BRL) according to manufacturer's instructions.

Chromosomal DNA on the slide was denatured in 70% formamide at 67°C for 2 min and dehydrated in 70%, 95% and 100% ethanol at -20°C for 5 min each. The probe mixture containing 50% formamide, 10% dextran sulphate, $2\times$SSC, 500 μg/mL salmon sperm DNA, 500 ng/mL 5S rDNA probe labeled with digoxigenin-11-dUTP and 500 ng/mL 18S-26S rDNA probe labeled with biotin-16-dUTP was heated at 100°C for 10 min and then kept on ice for 5 min. A 10 μL of the hybridization mixture was applied to each slide preparation, and the slides were covered with a coverslip. Slides were then placed in a humid chamber at 37°C for 6 h or longer. After hybridization, coverslips were removed in $2\times$SSC and the slides were washed in $2\times$SSC for 5 min, 50% formamide for 15 min at 37°C, $2\times$SSC for 15 min, $1\times$SSC for 15 min, and $4\times$SSC for 15 min that allowed binding of the probe with a minimal homology. The slides were covered with 65 μL of antidigoxigenin-rhodamine conjugate for digoxigenin-labeled 5S rDNA probe and avidin-FITC (Fluorescein isothiocyanate) conjugate for biotin-labeled 18S-26S rDNA probe mixture dissolved in 1% BSA/$4\times$SSC as detection buffer and incubated for 1 h at 37°C without coverslips. After slides were washed in $4\times$SSC for 10 min, $4\times$SSC/0.1% triton X-100 for 10 min, $4\times$SSC for 10 min and 2 X SSC for 5 min at room temperature. The final concentration (1 μg/mL) of 4',6-diamidino-2-phenylindole (DAPI) solution containing 1,4-Diazabicyclo-[2.2.2.]octane (DABCO) antifading solution as a counterstain, was added to amount of 25 μL on each slide and overlaid with a coverslip. The FITC was excited by light of 450-490 nm wavelength and the rhodamine was excited by light of 510-550 nm wavelength.

RESULTS

The bi-color fluorescence *in situ* hybridization with the digoxigenin-labelled pTa794 and the biotin-labelled pTa71, revealed six sites of 5S and four sites of 18S-26S rDNAs as shown in Fig. 1 with interphase (A) and somatic metaphase chromosomes (B) in *A. victorialis*. Six 5S hybridization sites were detected with rhodamine conjugated to antidigoxigenin as red dots against blue chromosome counterstained with DAPI and were localized on the three homologous intercalary region of short a... [illegible] homologous chromosome 6. ISH with the biotin-labeled 18S-26S rDNA probe, detected with FITC conjugated to avidin, revealed four sites on metaphase chromosomes. The signals of major NOR loci were detected on homologous chromosome 4, and two minor NOR loci on distal part of short arm of chromosomes 2 and 6, respectively. The major NOR loci of 18S-26S rDNA corresponded to the secondary constrictions including short flanking chromosomal segments on satellite and short arm. In interphase nuclei, as in meta-

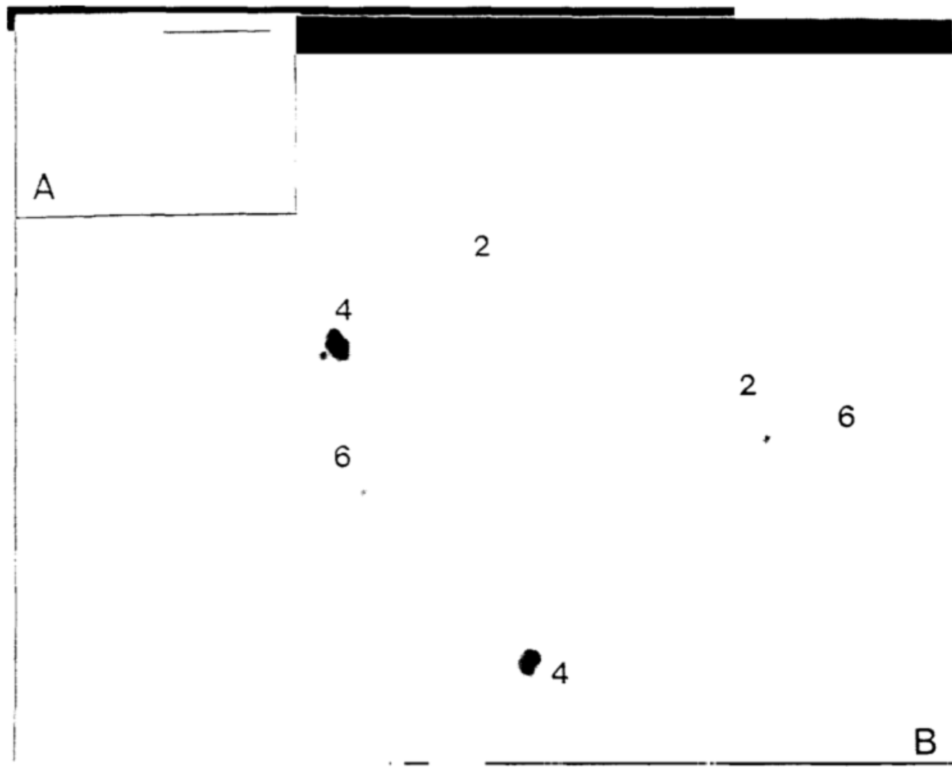

Fig. 1. FISH with the 5S and 18S-26S rDNA probes of interphase (A) and metaphase (B) cells in *A. victorialis*. 5S rDNA probes were labelled with digoxigenin and detected with antidigoxigenin-rhodamine (red color). 18S-26S rDNA probes were labelled with biotin and detected with avidin-FITC (green color).

phase, the same number of hybridization sites were detected. The minor 18S-26S rDNA signals were detected on telomeric region of short arm in chromosome 2 and 6 showing polymorphic pattern within genome. But 5S rDNA signals were accurately detected in homologous region of chromosome 6. Thus the 5S rDNA and 18S-26S rDNA signals distinctively appeared on short arm in only one chromosome 6. No association between the chromosomal location of major NORs and 5S sites was observed (Fig. 1B). However, the relative positions of two ribosomal RNA gene families were identified by FISH, and the 5S rDNA loci were linked with minor NORs in chromosome 6.

The banding method using fluorochrome were used to identify the relation between rDNA sites and CMA_3 positive heterochromatin. CMA_3, DNA-binding guanine-specific antibiotics, has been widely used as chromosome fluorescent dyes which binds to exposed GC-rich regions. CMA_3 staining in *A. victorilais* revealed bright bands corresponding only to the secondary constrictions of the satellite chromosomes, indicating that they are the most GC-rich regions in the chromosome. CMA_3 staining region identifies in the telomeric region of short arm that appears as a bright fluorescent zone in chromosome 4 as shown in Fig 2. In interphase nuclei, as in metaphase, the equal number of strong CMA_3 signals were detected. This region corresponds to the region of 18S-26S rDNA (Fig. 3), while CMA_3 positive bands does not appear in the region showing minor 18S-26S rDNA

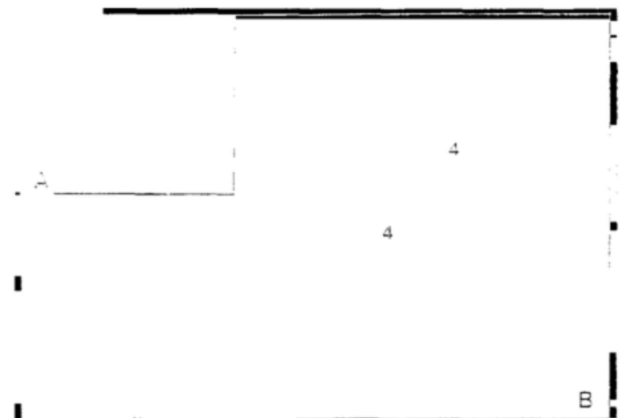

Fig. 2. CMA_3 banding of interphase (A) and metaphase (B) cells in *A. victorialis*.

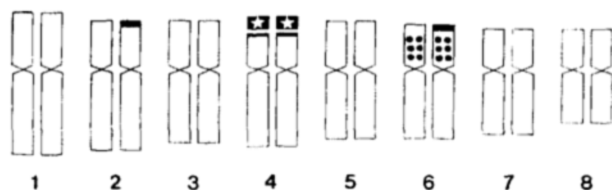

Fig. 3. Idiogram for chromosomal localization of 5S and 18S-26S rDNAs by FISH in *A. victorialis*. Circles indicate 5S rDNA and rectangles indicate 18S-26S rDNA loci. CMA_3 positive sites were represented with asterisks. Numbers indicate chromosome number.

sites of chromosomes 2 and 6. Thus, the major NOR sites contained CMA_3 positive heterochromatin in this study. This region corresponds to strongly C-banded heterochromatin (from data Kim *et al.*, 1990).

The results of CMA_3 fluorescent banding showed that not all the NORs are rich in GC content. Only 18S-26S rDNA was hybridized sites of chromosome 4 are consistent with CMA_3 positive bands in *A. victorialis*.

DISCUSSION

As in some *Allium* species (Schubert and Wobus, 1985; Ricroch *et al.*, 1992; Hizume, 1994a, 1994b), this study using FISH provides an efficient method for identifying the number and relative positions of 5S and 18S-26S rDNA loci. The distribution of both 5S and 18S-26S rRNA genes are independent arrays in most eukaryotes, while 5S rRNA genes are part of the 18S-26S rRNA genes arrays in yeast and certain fungi (Appels and Honeycutt, 1986).

The analysis of the chromosomes carrying both 5S and NOR loci in this species reveals that both loci are linked partly but independent arrays in chromosomes 2, 4 and 6 (Figs. 1 and 3). The signals of 18S-26S rRNA gene loci labelled with FITC were detected in the region of secondary constrictions flanked with satellite and distal end of short arm on chromosome 4. In addition minor 18S-26S rRNA genes loci were also detected in distal regions of chromosomes 2 and 6 showing heterozygous pairs as far as this signal concerned. Ekatherina *et al.* (1996) argued that the minor NORs probably were derived from major NORs and these minor sites do not usually form secondary constrictions, indicating that these regions are not transcribed. They suggested that the minor loci were often located in the telomeric regions of chromosomes and the major NOR locus of one chromosome either split into minor loci, or the locus corresponding to the major NOR was inactivated with the subsequent reduction in the number of rDNA units. The present study suggested the view that as in the present material as well the two positive signals were found exclusively at the end of the short arm of the 'standard' nucleolar chromosomes, which normally possess a small heterochromatic satellite. Movement of NORs between different chromosomes and the expression of additional NORs under extreme environmental conditions has been reported for several plant and animal species (Schubert and Wobus, 1985; Muratova and Sedelnikova, 1993). Schbert and Wobus (1985) reported that NORs in some onion species behave like mobile genetic elements. They suggested that NORs of some *Allium* chromosomes are free to jump at least between some preferential chromosomal sites, either by means of adjacent transposable elements or due to the presence of recombination hot spots in the terminal heterochromatin blocks of the short chromosome arms that find homologous sequences inside or proximal to the NORs. This conclusion was based on the variability in size, number and chromosomal position of active NORs and the number and size of interphase nucleoli after $AgNO_3$ staining in *Allium cepa* and *A. fistulosum*, and their interspecific hybrids. *A. victorialis* used in this study have been mainly propagated by vegetative propagation although some plants were propagated by seed formation. We suppose that some minor loci were derived from major NOR loci as transposable element during vegetative reproduction. A number of data make us believe that mobility of NORs is not confined to *Allium*. Additional minor 18S-26S rDNA loci were detected in the genome of some *Triticeae* species using *in situ* hybridization (Mukai *et al.*, 1991; Leitch and Heslop-Harrison, 1992; Jiang and Gill, 1994; Dubkovsky and Dvořák, 1995; Yamamoto and Mukai, 1995; Ekatherina *et al.*, 1996). And some authors reported on a new locus of 18S rRNA gene in chromosomes of a cultivated clone of *Allium sativum* in China (Hizume *et al.*, 1995), on variability of the number of nucleoli in *Crotalaria* (Verma and Raina, 1981), and on the occurrence of additional rDNA with NOR-forming ability in the intercalary heterochromatin of *Drosophila* (Ilyin *et al.*, 1978).

It has been proposed that the CMA_3-positive regions of the chromosomes relate to the rRNA genes (Schweizer, 1979; Sumner, 1990). C-bands could visualize all classes of heterochromatin in general while fluorescent bands could visualize base specific type of heterochromatin (data not shown). The CMA_3 positive bands corresponded to the only major region of 18S-26S rRNA genes.

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