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Physical Mapping by FISH and GISH of rDNA Loci and Discrimination of Genomes A and B in *Scilla scilloides* Complex Distributed in Korea

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The chromosomal locations of the 18S-26S (45S) and 5S rDNA loci in cytotypes AA, BB, and AABB of *Scilla scilloides* Complex from Korea were physically mapped using multicolor fluorescence *in situ* hybridization (McFISH). Genomic *in situ* hybridization (GISH) was also performed to distinguish between the AA and BB genomes in allotetraploid AABB plants. One 18S-26S rDNA locus was detected in both AA (a2) and BB (b1); one locus also was found in the allopolyploid AABB (b1). This demonstrated the loss of that locus in genome A. GISH with biotin-labeled DNA from the BB genome and digoxigenin-labeled 18S-26S rDNA probes revealed that the 18S-26S rDNA in AABB plants was localized in the nucleolus organizer region (NOR) of genome B. One and two 5S rDNA loci were found in diploids AA and BB, respectively. As expected, all three 5S rDNA loci were detected in the AABB plants. The sequence identities of the 5S rDNA genes among cytotypes AA and BB, AA and AABB, and BB and AABB were 99%, 95%, and 95%, respectively.

Keywords: FISH, genome, GISH, rDNA loci, *Scilla scilloides*

Scilla scilloides Complex, a bulbous perennial plant of Liliaceae, is widely distributed throughout Far East Asia, especially in mainland China, Korea, and Japan. Its chromosome complements comprise two types of well-differentiated genomes, A (x = 8) and B (x = 9). Based on those two basic genomes, various cytotypes have been found in natural populations: AA (2n = 16), AB (2n=17), BB (2n = 18), AAB (2n = 25), ABB (2n = 26), BBB (2n=27), AAAA (2n=32), AABB (2n = 34), ABBB (2n = 35), BBBB (2n = 36), AABBB (2n = 43), AAABBB (2n = 51), and AAAABBBB (2n = 68) (Morinaga, 1932; Okabe, 1938; Araki, 1972, 1985; Araki et al., 1976; Haga and Noda, 1976; Yu and Araki, 1991; Ding et al., 1998; Choi et al., 2004).

This species complex serves as a model by which plant cytogenetics can be used to study the differentiation and evolution of genomes due to the occurrence of distinct diploids and allo (or auto) polyploids. The homoeologous relationship between the A and B genomes in this species has been demonstrated by intergenomic pairing during meiosis (Araki, 1971; Noda, 1974). In addition to their karyotype differentiation, allopolyploid plants show variation in the nucleolar organizer regions (NORs) located in the secondary constriction of chromosomes (Haga and Noda, 1976). A reduction in the number or expression of NORs frequently occurs during polyploidization in *S. autumnalis* (Vaughan et al., 1993) and *Milium montanum* (Bennett and Thomas, 1991). This phenomenon is referred to as nucleolar dominance or amphiplasty (Navashin, 1928; Flavell, 1989).

Fluorescence *in situ* hybridization (FISH), which involves various molecular markers, e.g., rDNAs (45S and 5S), satellites, and other specific sequences, is an effective tool for detecting and analyzing genomic variation (Taketa et al., 1999; Jiang and Gill, 2006; Choi et al., 2007; Seo et al., 2007a). Genomic *in situ* hybridization (GISH), which uses genomic DNA as a probe, is another chromosome-mapping technique for detecting parental genomes in allopolyploids (Schwarzacher et al., 1989; Anamthawat-Jonsson et al., 1990; Leitch et al., 1990; Bennett et al., 1992; Lim et al., 2005).

In *S. scilloides*, the rRNA genes from plants distributed throughout Japan are localized (Hizume and Araki, 1994, 1997). Chromosomal *in situ* hybridization has revealed that allopolyploid plants possess NORs only on chromosome b1 in genome B but not in genome A. The 5S rRNA gene loci do not change in the genomes of allopolyploids, such as ABBB and AABB plants. Both genomes A and B can also be discriminated by GISH in allopolyploids ABBB and AABB (Hizume and Araki, 1996). The karyotype, C-banding pattern, and cytogeographic distribution of this species in Korean populations has also been investigated (Choi, 1996; Choi et al., 1997, 2004). However, little is known about the molecular cytogenetics of the species complex distributed in Korean natural populations.

Here, we used FISH and GISH techniques to analyze the genomic organization in *S. scilloides* (diploids AA and BB, and tetraploid AABB) distributed in Korea. We also compared the sequences for 5S rRNA genes from three cytotypes.

MATERIALS AND METHODS

Plant Material and Chromosome Preparation

Three cytotype plants of *Scilla scilloides* Complex, AA (2n = 16), BB (2n = 18), and AABB (2n = 34), were collected from Korean natural populations and used for chromosome preparation. Root tips, approximately 1 to 2 cm long, were excised, pre-treated for 3 h in a saturated solution of 1-bromonaphthalene, and fixed in 1:3 acetic acid-eth-

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anol (v/v). They were thoroughly washed, then macerated in an enzyme solution. This enzyme maceration/air-drying method was described previously (Fukui and Iijima, 1991).

Cloning and Sequencing of 5S rRNA Genes

Total genomic DNA was extracted from fresh leaves and purified according to the CTAB method (Graham et al., 1994). To clone the 5S rDNA fragments, PCR was performed with 2.5 units of *Taq* DNA polymerase, 0.5 μg of genomic DNA, and 1 μM of each primer set (5′-GATCCCATCAGAACTCC-3′ and 5′-GGTGCTTTAGTGCTGGTAT-3′) (Mochizuku et al., 1992). A 300-bp band of PCR products was excised and ligated into the pBluescript II KS(+) vector, then transformed into DH5α cells. DNA sequence data were analyzed by the BLAST network service at the National Center for Biotechnology Information (NCBI).

FISH and GISH

We directly labeled rDNA probes (18S-26S and 5S rDNAs) with biotin-16-dUTP and digoxigenin-11-dUTP by random primer labeling, according to the manufacturer's instructions (Roche). GISH probes were labeled with biotin-16-dUTP using the nick translation method (Roche).

FISH was performed as described previously (Choi et al., 2007). Chromosomal DNA on the slides was denatured in 70% formamide at 70°C for 2 min, then dehydrated in an ethanol series (70%, 95%, and 100%; 3 min each) at –20°C. The probe mixture (100 ng/mL probe DNA, 50% formamide, 10% dextran sulfate, 5 ng/μL salmon sperm DNA, and 2×SSC) was denatured at 99°C for 10 min and kept on ice for 5 min. This mixture (25 μL) was then added to the denatured chromosomal preparation, protected with cover slips, then sealed with rubber cement. After hybridization in a humid chamber at 37°C for 18 h, the cover slips were removed and the slides were washed twice in 2×SSC for 5 min. The 18S-26S and 5S probes were detected with avidin-FITC and anti-digoxigenin rhodamine (Roche), respectively, and the chromosomes were counterstained with 1 μg/μL DAPI (Vector Lab). Fluo-

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AA          GATCCCATCAGAACTCCGAAGTTAAGCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGA 60
AABB        GATCCCATCAGAACTCCGAAGTTAAGCGTGCTTGGGCGAGAGTAGTACTAGGATGGGCGA 60
BB          GATCCCATCAGAACTCCGAAGTTAAGCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGA 60
            ********************************************************** **

AA          CCTCCTGGGAAGTCCTCGTGTTGCACCCCTCTCTTTGTCGTTTTTCGAGGCCTCGATCAA 120
AABB        CCTCCTGGGAAGTCCTCGTGTTGCACCCCTCTCTTTGTCGTTTTTCGAGGCCTCGATCAA 120
BB          CCTCCTGGGAAGTCCTCGTGTTGCACCCCTCTCTTTGTCGTTTTTCGAGGCCTCGATCAA 120
            ************************************************************

AA          TTTGGGCCCTTATTTTTTAAATTTTTTCATACGGTTGCGAAGATCTCGAAAAGATCTTTC 180
AABB        TTTGGGGCCTTATTTTTTTAATTTTTTCGTACGGTTGCGAAGATCTCGAAAAAATCTTTC 180
BB          TTTGGGCCCTTATTTTTTAAATTTTTTCATACGGTTGCGAAGATCTCGAAAAGATCTTTC 180
            ****** *********** ********* ********************** *******

AA          CGTTGATATATTGCTCGCCCGAAACGGATCACCGAGCGACTATTTACGACGATTTCAAGT 240
AABB        CGTTGATATATTGCTCGCCCGAAACAAATCACCGAGCGACTATTTACGACGATTTCAAAT 240
BB          CGTTGATATATTGCTCGCCCGAAACGGATCACCAAGCAACTATTTACGACGATTTCAAGT 240
            ************************  ****** *** ******************** *

AA          CGAAACACGAAAGAATAAATAGAAAACGTCGAGGAGAGTTGACGGGTGCGATCATACCAG 300
AABB        CGAAACACGAAATAATGAATAGAAAACGTCGAGGCGAGTTGACGGGTGCGATCATACCAG 300
BB          CGAAACACGAAAGAATAAATAGAAAACGTCGAGGAGAGTTGACGGGTGCGATCATACCAG 300
            ************ *** ***************** ************************

AA          CACTAAAGCACCG 313
AABB        CACTAAAGCACCG 313
BB          CACTAAAGCACCG 313
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Figure 1. Sequence alignment of 5S rRNA genes from cytotypes AA, BB, and AABB of *Scilla scilloides*. Gray boxes represent coding regions.

rescence signals were detected with a Leica epi-fluorescence microscope (Leica, German), and the images were processed with a cooled CCD Camera (CoolSNAP, Photometrics) and Meta Imaging Series™ 4.6 software. The final printed images were prepared using Adobe Photoshop version 7.0.

RESULTS AND DISCUSSION

Sequence Analysis of 5S rRNA Gene

We cloned and compared the nucleotide sequences of the 5S rRNA genes that contained coding and non-coding regions in three cytotypes of *Scilla scilloides*: AA (2n = 16), BB (2n = 18), and AABB (2n = 34). PCR-amplification of the fragments showed three distinct bands with approximate sizes of 300-, 600-, and 900-bp repeating units. The 300-bp PCR products were directly cloned into the pBluescript II KS(+) vector for sequencing. All 5S rDNA sequences were 313 bp long and contained a 120-bp coding region and a 193-bp nontranscribed spacer (Figure 1). A high degree of sequence homology (99%) was found between diploids AA and BB. Sequence identities were 95% between cytotypes AA and AABB, and between cytotypes BB and AABB. In higher plants, the 5S rRNA genes are highly conserved and organized in tandem arrays, with coding regions of approximately 120 bp and variable non-coding spacers. Due to their variability, the spacer sequences are effective for studying inter- and intraspecific relationships in many plants (McIntyre et al., 1992; Volkov et al., 2001; Kim et al., 2006; Seo et al., 2007a, b).

FISH and GISH

To localize the rRNA genes in three cytotypes (AA, BB, and AABB) of *S. scilloides*, we simultaneously hybridized both probes of biotin-labeled 18S-26S rDNA and digoxigenin-labeled 5S rDNA. Results of the FISH and GISH analyses are presented in Figures 2 and 3. For cytotype AA (2n = 16), one 18S-26S rDNA locus was detected in the nucleolar organizer region (NOR) of chromosome a2, while the 5S rDNA locus was found on the short arm of chromosome a8 (Figure 2A). In diploid BB (2n = 18), one 18S-26S rDNA locus was detected on the NORs of chromosome b1, and two 5S rDNA loci were detected on the short arms of chromosomes b2 and b8 (Figure 2B). For tetraploid AABB (2n = 34), three pairs of 5S rDNA signals were detected in both genomes (Figure 2C), while only one pair of 18S-26S rDNA signals was found in the AABB plants (Figure 2D). Therefore, McFISH combined with GISH was used to identify the origin of NORs in cytotype AABB. FISH revealed that a pair of 18S-26S rDNA genes on the chromosome of cytotype AABB originated from diploid BB (Figure 2D). However, all of the 5S DNA loci localized on chromosomes a8, b2, and b8 in both AA and BB plants were detected in cytotype AABB (Figure 2C). In addition, two genomes, A and B, were clearly discriminated in the tetraploid AABB plant by GISH, with biotin-labeled genomic DNA from AA and BB plants (Figure 2C, D). Figure 3 shows an idiogram of the locations for 18S-26S rDNA, 5S rDNA, and C-bands on the chromosomes of those three cytotypes.

Various cytotypes, from diploid to octaploid (AA, BB, AAB, ABB, AABB, ABBB, AABBB, AAABBB, and AAAABBBB), have been found in Korean natural populations of *S. scil-*

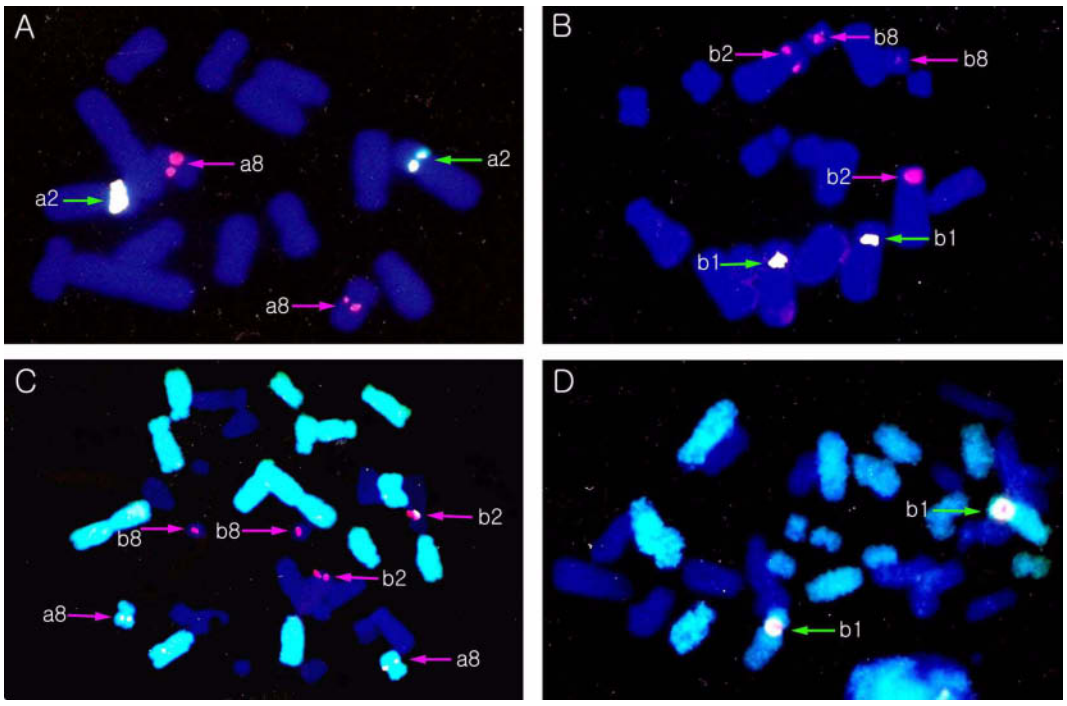

Figure 2. FISH and GISH patterns of cytotypes AA, BB, and AABB in *Scilla scilloides*. FISH of biotin-labeled 18S-26S rDNA (green arrows) and digoxigenin-labeled 5S rDNA (red arrows) of diploids AA and BB (**A**, **B**). GISH/FISH of biotin-labeled AA chromosomes (green color: GISH) and digoxigenin-labeled 5S rDNA (red arrows) in cytotype AABB (**C**). GISH/FISH of biotin-labeled BB chromosomes (green color: GISH) and digoxigenin-labeled 18S-26S rDNA (green arrows) in cytotype AABB (**D**).

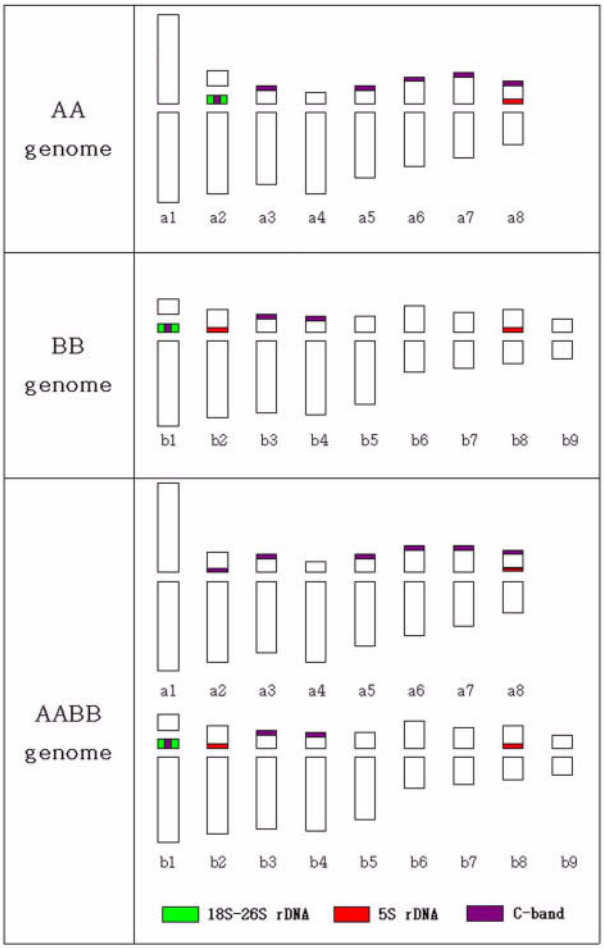

Figure 3. Idiogram of chromosomes from cytotypes AA, BB, and AABB, showing locations of rDNA sites (45S: green; 5S: red) in *Scilla scilloides*. C-bands (purple) are also marked (Choi et al., 1997).

loides (Araki, 1985; Choi et al., 2004). The allotetraploid AABB is the most common on the Korean peninsula. This species complex originated from mainland China and was transferred to Korea and Japan by human activity (Maekawa, 1944). A cytogeographical survey has demonstrated that the plants of AABB, which is thought to be a stable cytotype in natural populations, are the most frequent type in all three countries. In contrast, cytotype AA is restricted to China and Korea, while BB plants tend to be distributed in the southern part of China, Korea, and Japan. AA plants have not yet been found in Japan (Haga and Noda, 1976). Although many cytological and cytogeographical studies have been conducted on this species complex, little molecular cytogenetic research has been done to clarify the relationship between genomes A and B in this species complex from Korea. In Japanese populations, physical mapping of the 45 rRNA gene has been carried out in six cytotypes of *S. scilloides* via ISH (*in situ* hybridization), applying a cytochemical technique that involves the use of an avidin-horseradish peroxidase complex and diaminobenzidine tetrahydrochloride (DAB). Diploid plants have two rDNA sites on all NOR chromosomes (a2 and b1), while polyploids (cytotype ABB and AABB) have rDNA signals only on the B genome (b1) (Hizume and Araki, 1994). Our results from FISH mapping of rDNA (18S-26S and 5S) did not differ from previous cytochemical data obtained from plants distributed in Japan. It is important to note that some of the Japanese AABB plants had all four NOR sites on genomes A and B. Deletion of the NOR in one genome occurs in some amphidiploid species that originates from a combination of different genomes, and its activity is suppressed (Flavell, 1989; Vaughan et al., 1993). Here, we observed the same type of nucleolar dominance in cytotype AABB, showing the deletion of the NOR in genome A. Elimination of the rRNA genes in chromosome a2 might have happened through unequal crossing-over (Ritossa, 1973).

GISH conducted with a genomic DNA probe is very useful for investigating the constitution, origin, and evolution of a genome, especially in hybrid plants and cultivated polyploids. Successful applications of this technique have been reported in allopolyploids *Triticum* (Mukai et al., 1993), *Nicotiana* (Lim et al., 2005), and *Brassica* (Ge and Li, 2007). In our study, chromosomes from genomes A and B could also be clearly discriminated by GISH in the AABB plants (Figure 2C, D). This diversification of both genomes might be due to genome homogenization, especially in the repetitive sequences, during their evolutionary process (Dover, 1982). Previously, Araki (1971) reported that both genomes have well-differentiated karyotypes with a homoeologous relationship, and that genome A was derived from genome B through translocation, inversion, and centromeric deletion. Translocation between the genomes has been identified by GISH in an ABBB plant distributed throughout Japan (Hizume and Araki, 1996).

Therefore, it is very likely that both homoeologous genomes A and B of this species complex show different patterns of genomic evolution in terms of their adaptation and polyploidization in natural populations. Our FISH/GISH data support previous cytological data and demonstrate the organization and relationship between genomes A and B.

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