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Karyotypes of Three Somaclonal Variants and Wild Plants of *Allium tuberosum* by Bicolor FISH

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Using the fluorescence in situ hybridization (FISH) technique, we conducted karyotype analyses to identify the lost chromosomes in three somaclonal variants obtained from tissue culture of wild *Allium tuberosum* (2n = 4X = 32). The three lost chromosomes of the At29 variant (2n = 29) were all chromosome 2, the two for At30 (2n = 30) were chromosomes 7 and 8, and At31 was missing chromosome 2. Chromosome compositions of these variants were confirmed as being fixed lines during two years of greenhouse cultivation. The bicolor FISH technique, involving both 5S and 18S-5.8S-26S ribosomal RNA genes as probes, was used to assign chromosomal locations and to confirm whether the lost chromosomes contained any rRNA markers. The 5S rRNA gene signals in all variants as well as the wild type were detected as two sets, one on the intercalary region of the short arm of chromosome 3, the other on the intercalary region of the long arm of chromosome 6. One 18S-5.8S-26S rRNA gene site on the secondary constriction included a flanking satellite and terminal region on the short arm of chromosome 8. Signals of the 18S-5.8S-26S rRNA gene in At30 showed in only three chromosomes, indicating that one of the lost chromosomes was chromosome 8. Overall, three marker chromosomes were established by FISH, using rRNA multigene families.

Keywords: *Allium tuberosum*, chromosomal location, FISH, rRNA gene

Allium tuberosum (2n = 4X = 32) is a unique autotetraploid species among the genus cultivated in Korea. Using conventional karyotype and Giemsa C-banding patterns, Seo (1977) showed that the basic genome of *A. tuberosum* consisted of seven metacentric chromosomes (no. 1-7) and one subtelocentric chromosome containing a satellite (no. 8). However, the C-banding patterns did not adequately identify each metacentric chromosome because constitutive heterochromatin was poorly distributed in both terminal regions.

Recently, the fluorescence in situ hybridization (FISH) technique has been successful for identifying marker chromosomes and physical mapping in many species. Probes of repeated sequences and multigene families, including rRNA genes, have been used (Lapitan et al., 1989; Maluszynska and Heslop-Harrison, 1991; Mukai et al., 1991; Tsujimoto and Gill, 1991; Anamthawat-Jónsson et al., 1993; Leitch et al., 1993; Jiang and Gill, 1994). The physical mapping of repeated sequences in *Allium* species also has been widely reported (Irifune et al., 1995; Seo et al., 1999) and rRNA genes (Ricroch et al., 1992; Hizume, 1994; Lee and Seo, 1997; Seo et al., 1997; Lee et al., 1998).

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Ploidy level in somaclonal variations for plants is classified as being one of two types: polyploid or aneuploid (Roy, 1980; Seo and Kim, 1988; Müller et al., 1990). Tissue culture has been carried out in many *Allium* species for in-vitro mass production or crop improvement. We have reported the frequency of several kinds of somaclonal variants, e.g., At28, At29, At30, At31, and At33 (2n = 28, 29, 30, 31, and 33, respectively), as obtained from tissue culture of wild *A. tuberosum* (Do et al., 1999). Among these, At28 and At33 withered within one year under soil conditions.

In the present study, we applied FISH to wild *A. tuberosum* and three somaclonal variants, At29, At30, and At31. Our objectives were:

(1) to analyze the karyotypic change in three somaclonal variants compared with the wild type, using 5S and 18S-5.8S-26S rRNA genes as probes; and

(2) to confirm by FISH marker and conventional staining analysis whether the lost chromosome contained any rRNA genes.

MATERIALS AND METHODS

Conventional Staining Analysis

Somaclones identified as aneuploids were main-

tained for two years in the greenhouse. Actively growing root tips of wild *A. tuberosum* and three somaclonal variants, At29, At30, and At31, each approximately 1 cm long, were pretreated with 2 mM 8-hydroxyquinoline for 6 h at 16°C, and fixed in acetic-ethanol (1:3) at 4°C overnight. The samples were then hydrolyzed for 30 s at 60°C in 1N HCl. Afterward, squash mounts were prepared with aceto-orcein. Thirty metaphases were analyzed from a wild-type and three variants.

Chromosome Preparation

For the FISH analysis, metaphase preparations were made, following slightly modified procedures from Mukai et al. (1990). Freshly sampled root tips were pretreated in ice water (0°C) for 21 h and fixed in acetic-ethanol (1:3) for 4 d at room temperature. The root tips were stained in a 1% aceto-carmine solution for 1 h, then squashed in 45% acetate. After the cover glasses were removed via freezing, the slides were destained by immersing them in 45% acetate at room temperature. The air-dried slides were maintained in a desiccator.

Probe Labelling

Probe pTa71 contained a 9-kb EcoRI fragment of the 18S-5.8S-26S rDNA that was derived from common wheat (*Triticum aestivum* cv. Chinese Spring; Gerlach and Bedbrook, 1979) and then re-cloned in pUC18. This probe was labelled with biotin-16-dUTP (Boehringer Mannheim) via the nick translation method, according to manufacturer's instructions (Gibco BRL). pTa71 was kindly supplied by Dr. Mukai, Dept. of Biological Science, Osaka Kyoiku University. The probe pTU602 contained a 540-bp insert comprising the conserved 120-bp of the 5S rRNA coding region and a non-transcribed sequence isolated from wild *A. tuberosum*. This probe was labelled with digoxigenin-11-dUTP (Boehringer Mannheim), via the following PCR: the optimum reaction mixture contained 5 ng wild *A. tuberosum* template DNA; 5 pM of forward (5'-GATCCCATCAGAACTCC-3') and reverse (5'-GGTGCTTTAGTGCTGGTAT-3') primers; 0.2 mM each of dATP, dCTP, and dGTP; 0.06 mM dTTP; 0.14 mM digoxigenin-11-dUTP; and 2.5 U Ex *Taq* DNA polymerase (Takara Shuzo Co. Ltd) in a 100 µL Ex *Taq* buffer (Takara Shuzo Co. Ltd) with 2 mM magnesium chloride. The PCR reaction was performed in a Perkin-Elmer Cetus System 9600 thermal cycler. Amplification was achieved using a series of preliminary 2-min denaturations at 94°C; 30 cycles of 1 min at 94°C, 1 min at 55°C (anneal) and 2 min at 72°C (extension); and a final extension at 72°C for 10 min, then slowly cooling to room temperature.

Bicolor Fluorescence in Situ Hybridization

FISH was made using procedures described by Seo et al. (1997). Chromosomal DNA on the slides was denatured in 70% formamide at 67°C for 2 min, then dehydrated in a 70%, 95%, and 100% ethanol series at −20°C for 5 min each. The probe mixture contained 50% formamide (w/v), 10% dextran sulfate (w/v), 5 ng/µL salmon sperm DNA, 500 ng/mL of digoxigenin-labelled 5S rRNA gene probe and biotin-labelled 18S-5.8S-26S rRNA gene probe. This mixture was heated at 100°C for 10 min and kept on ice for 5 min. Ten µL of the probe mixture was applied to each denatured preparation and covered with glass. Slides were then placed in a humid chamber at 37°C for 6 h.

After hybridization, the cover glasses were removed in 2X SSC. The slides were then washed in 2X SSC for 5 min, 50% formamide for 15 min at 37°C, 2X SSC for 15 min, 1X SSC for 15 min, and 4X SSC for 15 min for binding of the probe with minimal homology. They were covered with 65 µL of an antidigoxigenin-rhodamine conjugate for the digoxigenin-labelled 5S rRNA gene probe and an avidin-FITC (fluorescein isothiocyanate) conjugate for the biotin-labelled 18S-5.8S-26S rRNA gene probe mixture. These were dissolved in 1% BSA/4X SSC and incubated for 1 h at 37°C without cover glasses. The slides were then washed in 4X SSC for 10 min, 4X SSC/0.1% Triton X-100 for 10 min, 4X SSC for 10 min, and 2X SSC for 5 min. The final concentration of 1 µg/µL of 4,6-diamino-2-phenylindole (DAPI) solution, containing Vectashield anti-fading solution (Vector) as a counterstain, was added to 15 µL of sample on each slide and overlaid with a cover glass. Signals were detected with a Carl Zeiss epifluorescent microscope that was equipped with filter sets: No. 15 for rhodamine, No. 09 for FITC, and No. 02 for DAPI. Photographs were taken with Kodak superclear 400 color film.

RESULTS

Somaclones identified as aneuploids were maintained for two years in the greenhouse. These variants showed that growth and viability were similar to that of the wild *A. tuberosum*. In the conventional staining

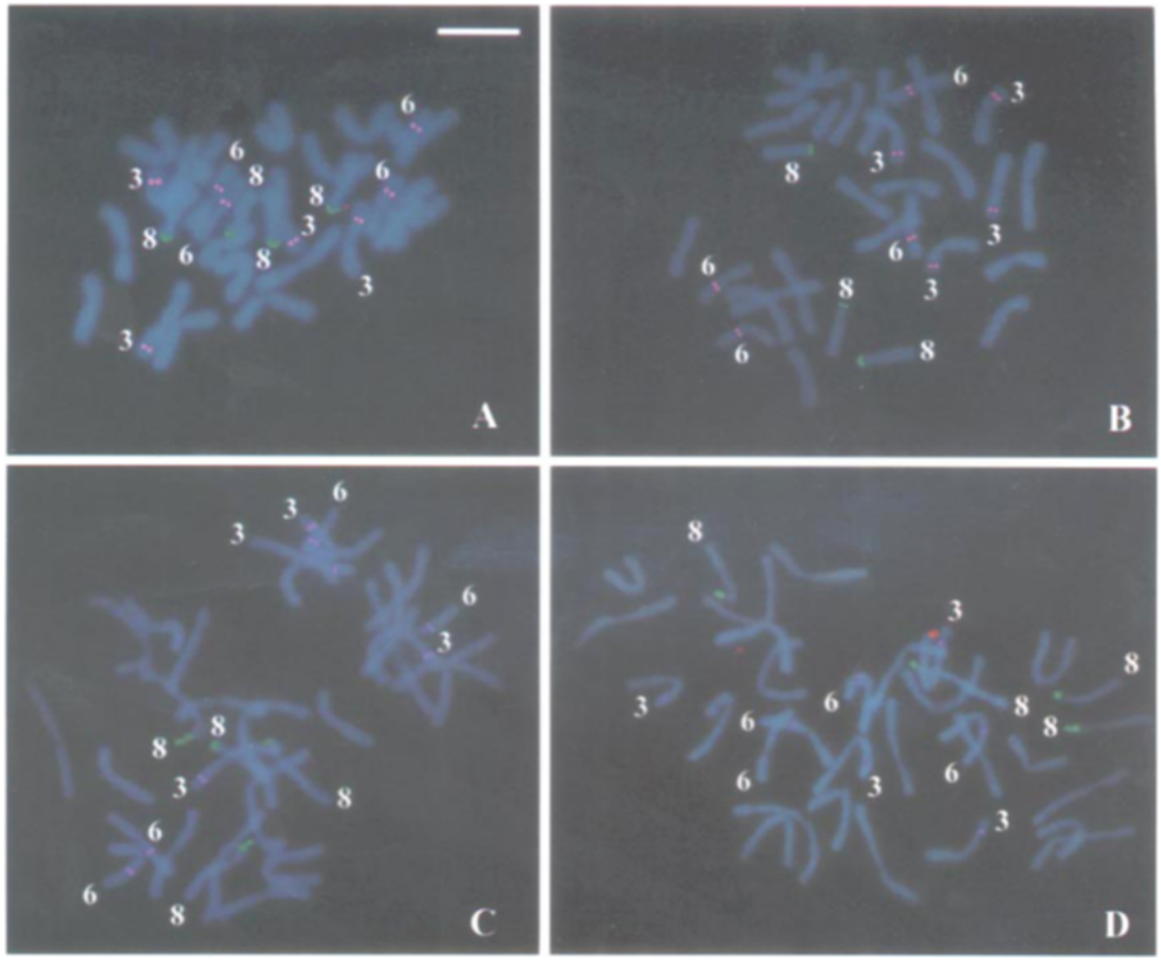

Figure 1. Bicolor FISH patterns on the metaphases of At29 (A), At30 (B), At31 (C), and wild type (D) in *A. tuberosum* using both 5S and 18S-5.8S-26S rDNA probes (bar = 10 μm). Digoxigenin-labelled 5S rDNA probe detected with antidigoxigenin-rhodamine conjugate (red) and biotin-labelled 18S-5.8S-26S rDNA probe detected with avidin-FITC conjugate (green). Numbers indicate corresponding chromosomes showing rDNA hybridization sites.

analysis, At29 lost three copies of chromosome 2, At30 one copy each of chromosomes 7 and 8, and At31 one copy of chromosome 2 (data not shown).

To confirm whether the lost chromosomes were marker chromosomes of the 5S or 18S-5.8S-26S rRNA genes, and to identify the lost chromosomes more precisely, we applied the bicolor FISH technique to wild-type *A. tuberosum* and three variants, At29, At30, and At31, using both rRNA gene probes. The bicolor FISH patterns of the digoxigenin-labelled 5S rRNA gene (red) and the biotin-labelled 18S-5.8S-26S rRNA gene (green) signals are shown for At29 (Fig. 1A), At30 (Fig. 1B), At31 (Fig. 1C) and the wild type (Fig. 1D).

5S rRNA signals were detected in two sites for all variants. One was localized in the intercalary region of the short arm of chromosome 3, the other in the intercalary region of the long arm of chromosome 6. The 18S-5.8S-26S rRNA signals were detected on the only site of chromosome 8 in which was localized the secondary constriction region that contained the flanking terminal portion of the short arm and satellite. Signals in At29 and At31 were the same as those for wild-type *A. tuberosum*, but those in At30 differed, having only three signals instead of four. Therefore, one of the two lost chromosomes in At30 was confirmed to be chromosome 8. This agreed with our conventional staining results. Figure 2 shows the idiograms on the FISH patterns, using multigene families of the 5S and 18S-5.8S-26S rRNA genes as probes in

wild-type *A. tuberosum* and three variants.

DISCUSSION

The success of crop improvement programs via tissue culture depends on controlling the variation in chromosome number and structure during in-vitro culture (Sekerka, 1977; Larkin and Scowcroft, 1981; Nagarajan and Walton, 1987). Plantlets derived from callus culture that were aneuploid in somatic chromosome number were maintained in the greenhouse to improve breeding performance. Among the many aneuploid types obtained in this study, two (At28 and

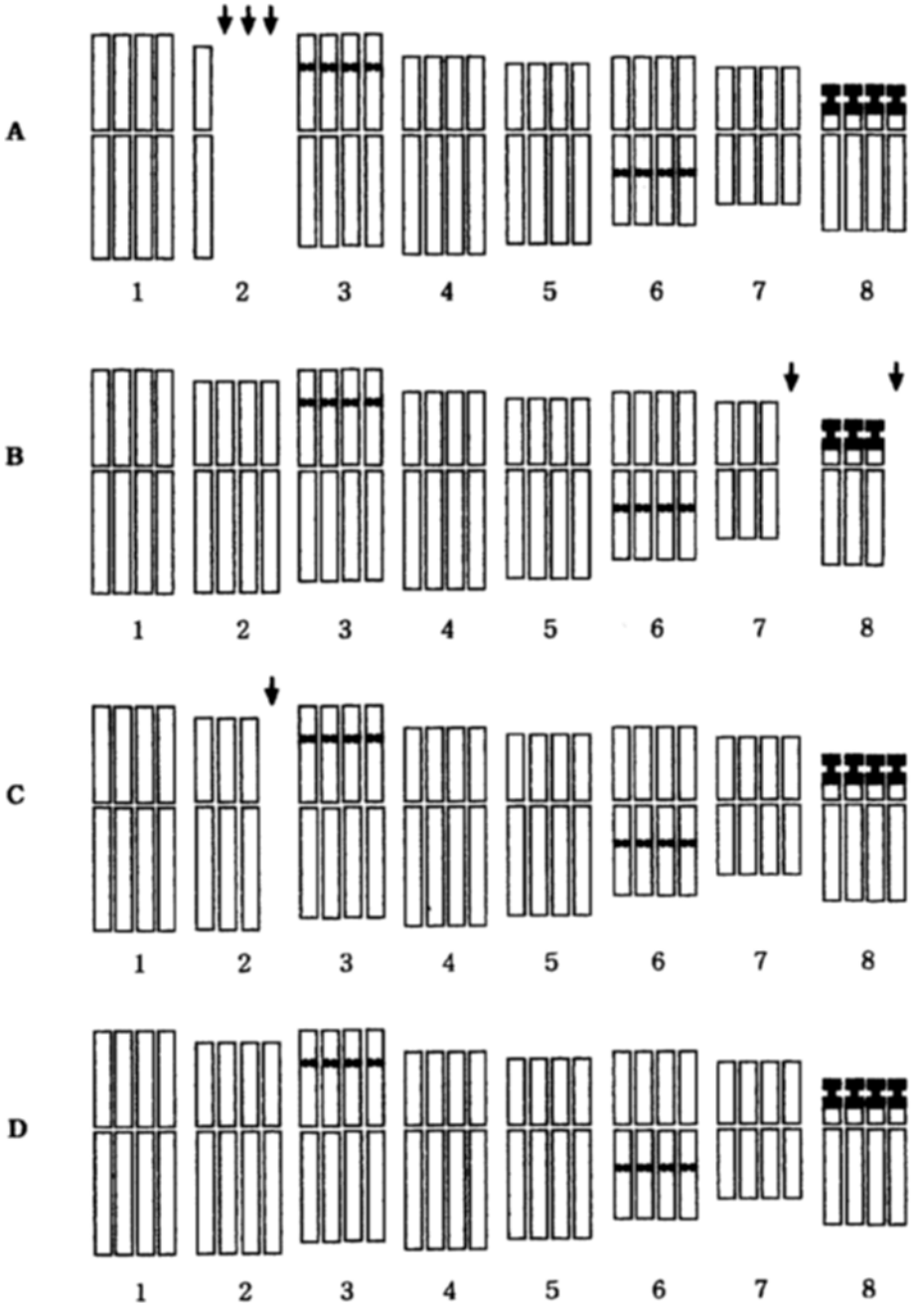

Figure 2. Idiogram for chromosomal localization of 5S and 18S-5.8S-26S rDNAs in somaclones At29 (A), At30 (B), At31 (C), and wild type (D) of *A. tuberosum* observed by FISH. Circles indicate 5S rDNA and rectangles indicate 18S-5.8S-26S rDNA loci. The arrows indicate the lost chromosomes that are responsible for the abnormal karyotype.

At33) were not viable under soil conditions, but three variants, At29, At30, and At31 were well adapted to soil conditions for two years. These three showed considerable fitness under greenhouse cultivation. The frequency of At30 was highest (26%) among the somaclones. However, no variant recovered its original genome. Therefore, to investigate their karyotypic changes, we focused on the detailed conventional karyotypes and physical mapping by FISH, using 5S and 18S-5.8S-26S rRNA genes as probes.

To identify whether the number and position of the 5S and 18S-5.8S-26S rRNA gene loci in each variant were consistent with the wild type, bicolor FISH was applied to each metaphase chromosome of the three variants. Some authors have reported that the 18S-5.8S-26S rRNA multigene family, as a component of the nucleolar organizing region (NOR), is strongly hybridized in the secondary constriction and satellite (Mukai et al., 1991; Hizume, 1994; Castilho and Heslop-Harrison, 1995; Hizume et al., 1995; Irifune et al., 1995; Lee and Seo, 1997). In addition, although a somatic chromosome contains the sat-chromosome having the NOR site, the signal of the 18S-5.8S-26S rRNA gene is detected in another chromosome and the B chromosome (Seo et al., 1997; Lee et al., 1998). The 18S-5.8S-26S rRNA signal in *A. tuberosum* is detected in the secondary constriction and satellite of chromosome 8. Although satellite identification by conventional staining is very difficult because of its small and fragile constriction site, the FISH signal is easily detected.

Chromosomal location of the 5S rRNA genes varies by species and is independent of the 18S-5.8S-26S loci. Lee et al. (1999) reported that the 5S rRNA loci are used as a marker for relationship implications in some *Allium* species. Chromosomal location of the 5S rRNA genes in *A. tuberosum* was identified in two loci: one in the intercalary region of the short arm of chromosome 3, the other in the intercalary region of the long arm of chromosome 6. This pattern of chromosomal location for 5S rRNA genes in *A. tuberosum* is unique compared with those of other *Allium* species.

Three somaclonal variants had the same eight 5S rRNA gene sites as the wild-type *A. tuberosum* (Fig. 2, A-D). The shared number of 18S-5.8S-26S rRNA signals was four for At29, At31 and the wild plant (Fig. 2, A, C, and D), but three signals were different between At30 and the others (Fig. 2B). The lost chromosome of At29 and At31 was chromosome 2, for which rDNA sites were not detected (Fig. 2, A and C). The only lost chromosome of At30 was chromosome 8, with the 18S-5.8S-26S rDNA site (Fig. 2B). This suggests that, regardless of the presence of the rRNA genes, the lost chromosomes did not affect the viability of *A. tuberosum*.

The Bi- or multicolor FISH technique, using rRNA multigene families and other detectable DNA sequences as probes, will be very useful for determining the marker chromosome within somatic chromosomes that are similar in size and morphology among species. In this study with *A. tuberosum*, we established that chromosomes 3 and 6 were marker chromosomes of the 5S rRNA gene, and that chromosome 8 was a marker chromosome of the 18S-5.8S-26S rRNA gene. We also found that the application of FISH to somaclonal variants is a useful tool for identifying and understanding chromosomal changes during the tissue-culture process.

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