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# Analysis of somaclonal variation through tissue culture and chromosomal localization of rDNA sites by fluorescent *in situ* hybridization in wild *Allium tuberosum* and a regenerated variant

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**Abstract**

The effects of basal media and growth regulators on callus initiation and shoot regeneration have been investigated in wild *Allium tuberosum* (2*n* = 4*x* = 32). Callus initiation was greatest from flower bud explants cultured on MS medium supplemented with 2,4-D and BA at 1 mg $l^{-1}$ each. Maximum number of shoots was obtained from callus grown on MS medium supplemented with NAA and BA at 0.2 and 2 mg $l^{-1}$, respectively. The chromosome analysis of regenerants derived from callus revealed variation in ploidy, such as 2*n* = 28, 29, 30, 31, 33 as well as normal tetraploid. During the culture period for two generations, one aneuploid regenerant with 2*n* = 30 (named At30) showed better viability and growth than tetraploid plants and other aneuploid variants. In a karyotypic analysis of At30, the chromosomal positions of 5S and 18S-5.8S-26S rDNA were physically mapped by fluorescent *in situ* hybridization and compared to chromosomes of wild type *A. tuberosum*. Both wild type *A. tuberosum* and At30 exhibited two sets of 5S rDNA sites, one on the proximal position of the short arm of chromosome 3, and the other on the intercalary region on the long arm of chromosome 6. There was one 18S-5.8S-26S rDNA site in the secondary constriction including flanking short chromosomal segments of satellite and terminal regions on the short arm of chromosome 8 in wild type *A. tuberosum*. However, At30 showed only three labelled chromosome 8 indicating that this was one of the lost chromosomes of At30.

*Abbreviations:* BA – 6-benzyladenine; 2,4-D – 2,4-dichlorophenoxyacetic acid; 2ip – 2 isopentenyladenine; KIN – kinetin; MS – Murashige and Skoog (1962); NAA – 1-naphthalene-acetic acid

## Introduction

*Allium tuberosum* Rottl. ex. Spreng. is widely cultivated in South Asia, including Korea, Japan and China. The leaves of thin non-bulbous species have a characteristic flavor of genus *Allium*, alliin, and are used as vegetable. Both cultivated and wild accessions of *A. tuberosum* have the same chromosome number but minor phenotypic differences exist between them. Moreover, the wild genotype is suitable for culinary purpose because it has flavor of alliin.

Somaclonal variants in plants produced in tissue culture can be used to generate new cultivars (Müller et al., 1990; Linacero and Vazquez, 1992). Two general types of ploidy variation, polyploidy and aneuploidy, are frequently found in tissue cultured cells (Roy, 1980). Among factors influencing the frequency and spectrum of somaclonal variation, growth regulators play an important role in the induction of numerical changes of chromosomes (Nair and Seo, 1995). Tissue culture has been carried out in many *Allium* species for *in vitro* mass production, development of polyploids, and *in vitro* conservation. Despite the major position of *A. tuberosum* as vegetable, little is known about its performance in tissue culture. Reports concern plantlet regeneration from calli (Zee

et al., 1977), somaclonal variation in callus tissue (Roy, 1980), and *in vitro* propagation by direct shoot proliferation (Pandey et al., 1992).

*A. tuberosum* ($2n = 4x = 32$) is an autotetraploid consisting of seven sets of metacentric and one set of submetacentric chromosomes with satellites. Seo (1977) reported that this species has a few C-bands in the satellite and terminal regions of some chromosomes. Thus, it is difficult to identify each chromosome. However, fluorescent *in situ* hybridization (FISH) has been used in many plants to identify chromosomes accurately, using species-specific repetitive sequences, ribosomal genes, and even unique sequences (Mukai et al., 1990; Maluszynska and Heslop-Harrison, 1991; Jiang and Gill, 1994). Many authors reported the physical map of multigene families such as 5S and 18S-5.8S-26S rDNA and highly repeated DNA sequences (Lapitan et al., 1989; Mukai et al., 1991; Leitch and Heslop-Harrison, 1993). In some *Allium* species, chromosomal localization of these multigene families was detected by FISH (Ricroch et al., 1992; Hizume, 1994; Lee and Seo, 1997; Seo et al., 1997; Lee et al. 1998). However, no information is available about the chromosomal location of these genes in *A. tuberosum*. The physical location of these sequences can be useful for identifying the corresponding chromosome because *A. tuberosum* shows similar chromosome size and a few C-bands.

In this paper, we applied FISH to the wild *A. tuberosum* and At30, a somaclonal variant. The specific aims of the present investigation were:

(1) to generate plants from callus derived from flower bud explants of *A. tuberosum*; and
(2) to analyze the karyotypic change of At30 in comparison with the wild type by using 5S and 18S-5.8S-26S rDNA as probes.

## Materials and methods

### *Tissue culture and cytological analysis*

Plants of wild-type *A. tuberosum* collected at an altitude of about 1000 m in Kangwon province of Korea were maintained at local farm in the suburbs of Taegu city. Flower buds of wild-type *A. tuberosum* were rinsed in 70% (v:v) ethanol for 5 min, followed by surface sterilization in 5% (w:v) sodium hypochlorite solution for 20 min with vigorous agitation. They were then rinsed three times with sterilized deionized water. To induce callus formation, explants were placed on MS (Murashige and Skoog, 1962) medium with growth regulator combinations as in Table 1. The pH was adjusted to 5.8 with 1N HCl prior to autoclaving. All media were solidified with 0.8% Difco Bacto agar. Media were poured into 50-ml Erlenmeyer flasks containing 20 ml media. Twenty flasks with three explants each were cultured per treatment. The experiments were run twice. The cultures were incubated in the dark at 25°C. Callus obtained from all media was subcultured at 30-day intervals on the same medium for 120 days. At the time of each subculture, a part of vigorously growing callus obtained from MS medium supplemented with 2,4-D and BA at 1 mg $l^{-1}$ each was transferred randomly to shoot induction media with growth regulator combinations as in Table 2. Flasks were kept under continuous illumination (2000 μmol $m^{-2}$ $s^{-1}$) by cool white fluorescent light. Regenerated shoots (about 5 cm) were isolated each month up to 6 months and rooted on basal MS medium without growth regulators. Plantlets developing roots were transferred to sterilized vermiculite and perlite (1:1) and kept at room temperature under a 16-h photoperiod. The rooted plantlets were transferred to soil when fresh leaves appeared. Fresh weight (after 24 h at 80°C) was evaluated on five plants per treatment after 180 days of culture, collected at random from several pots. The ploidy was ascertained by counting the number of chromosomes in root-tips of the plantlets using the procedure of Seo et al. (1989).

### *Fluorescent in situ hybridization (FISH)*

pTa71, containing a 9-kb *Eco*RI fragment of the 18S-5.8S-26S rDNA derived from *Secale cereale* (Gerlach and Bedbrook, 1979) and re-cloned in pUC18, was labelled with biotin-16-dUTP by the nick translation method according to the manufacturer's instructions (Enzo Diagnostics, Farmingdale, NY). pTa71 was kindly supplied by Dr Mukai, Dept. of Biological Sciences, Osaka Kyoiku University. The probe pTU602, containing a 540-bp insert for a 120-bp tandem repeated 5S rDNA sequence isolated from wild *A. tuberosum*, was labelled with digoxigenin-11-dUTP by the polymerase chain reaction (PCR) as follows. The optimum reaction mix 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 100

μl Ex *Taq* buffer (Takara Shuzo Co. Ltd.) containing 2 mM magnesium chloride. The PCR reaction was performed in a Perkin-Elmer Cetus System 9600 thermal cycler. Amplification was achieved as follows: a preliminary 2-min denaturation at 94°C; 30 cycles of 1 min at 94°C (denaturation), 1 min at 55°C (anneal) and 2 min at 72°C (extension); and a final extention at 72 °C for 10 min followed by a slow cooling to room temperature.

Chromosome preparation and FISH experiments were made using procedures described by Seo et al. (1997). For detection of hybridization signals, we used Carl Zeiss epifluorescent microscope equipped with the filter sets No. 15 (Rhodamine), 09 (FITC), and 02 (DAPI). Photographs were taken on Kodak super clear 400 color film.

## Results

### *Tissue culture and cytological analysis*

Flower buds showed different frequency of callus formation (Table 1). Calli were induced 45 – 50 days after culture from all media regardless of growth regulators. Callus and regenerated shoots developed simultaneously on MS medium supplemented with 2,4-D, BA, and KIN at 2 mg $l^{-1}$each. Maximum frequency of callus initiation was obtained with 2,4-D and BA at 1 mg $l^{-1}$each. Vigorously growing callus obtained from this medium was plated onto nine kinds of shoot induction media as showed in Table 2. Shoots were produced from the surface of the calli about 1–2 weeks after transferring callus to the regeneration media. The first shoot induction was observed 130 days after culture on MS medium with NAA and BA at 0.2 and 2 mg $l^{-1}$ each. This medium also exhibited the highest shoot production. The number of shoots formed on MS medium with 2,4-D and BA at 0.2 and 2 mg $l^{-1}$ respectively was the lowest among the media tested (Table 2). Individual shoots (5 cm long) were easily rooted on growth regulator-free MS medium within 4 – 5 weeks of subculture. The roots formed directly at the base of the shoots with no additional callus. A total of 244 plantlets was transplanted to small plastic pots filled with a vermiculite perlite mixture and maintained at room temperature for 30 days. Surviving plantlets were easily adapted to soil.

Among 163 regenerants, 100 (61.3%) were normal tetraploid ($2n = 32$) and 63 (38.7%) were variants including 61 (37.4%) hypotetraploids and two (1.2%) hypertetraploids (Table 2). The frequency of regeneration of aberrant plants was highest (29%) with NAA and BA at 0.2 and 2 mg $l^{-1}$, respectively. We designated variants with chromosome composition of $2n$ = 28, 29, 30, 31, and 33 as At28, At29, At30, At31, and At33 respectively according to material used and chromosome number. Among these variants, At30 revealed the highest percentage of aneuploid cells (26.4%). One of the most interesting findings was the fact that At30 showed better viability and growth than wild-type plant and other aneuploid plants during the culture period for two generations. At30 was phenotypically normal, developed extensive roots and grew into healthy plants with normal flowering (data not presented). Moreover, fresh weight after 4 weeks of growth in the green house of five samples of the At30 ($4.4 \pm 0.6$ g/plant) was greater than that of wild type ($2.6 \pm 0.7$ g/plant).

*Table 1.* Percentage of flower bud explants initiating callus on MS media with several combinations of growth regulators

| Treatment (mg $l^{-1}$ ) | MS [a] |
|---|---|
| 1 2,4-D + 1 BAP | $88 \pm 0$ |
| 2 2,4-D + 1 BAP | $65 \pm 3$ |
| 2 2,4-D + 2 NAA + 2 BAP | $47 \pm 6$ |
| 2 2,4-D + 2 NAA + 2 KIN | $38 \pm 1$ |
| 2 2,4-D + 2 BAP + 2 KIN | $25 \pm 3$ [b] |

Twenty flasks with three explants each were cultured per treatment.
[a] Mean values of two experiments are given, ± standard error. $p \leq 0.05$.
[b] Simultaneous formation of callus and shoot.

### *Fluorescent in situ hybridization (FISH)*

Conventional staining analysis in both wild-type *A. tuberosum* and At30 identified lost chromosomes in At30 as one copy each of chromosomes 7 and 8 (data not shown). Identification of the lost chromosome was confirmed by *in situ* hybridization. We analyzed the chromosomal positions of the 5S and 18S-5.8S-26S rDNA sites by FISH using digoxigenin-11-dUTP-labelled 5S rDNA and biotin-16-dUTP labelled 18S-5.8S-26S rDNA as probes in wild-type *A. tuberosum* and At30. In wild-type *A. tuberosum*, two 5S rDNA sites were detected, on both median chromosomes 3 and 6 (Figure 1A). One site was on the proximal position of the short arm of chromosome 3, while the other on the intercalary region on the long

*Table 2.* Effects of growth regulators on regeneration of plantlets and somatic variants formation from flower bud explants on MS medium. Shoot was induced from 2 g of callus cluster per treatment

| Treatment (mg $l^{-1}$) | | No. of shoots (*in vitro*) | No of regenerants in soil | No of hypotetraploids (%)[c] | | | | No of tetraploids (%)[c] | No of hypertetra-ploids (%)[c] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 2*n* = 28 | 2*n* = 29 | 2*n* = 30 | 2*n* = 31 | 2*n* = 32 | 2*n* = 33 |
| 0.2 2,4-D | 2 2ip | 13 (5/6/2) [a] | 6 (2/4/0)[b] | | | | | 6 | |
| | 2 BAP | 2 (1/1) | 1 (1/0) | | | | | 1 | |
| | 2 KIN | 38 (8/14/12/3/1) | 23 (7/10/4/1/1) | | | 2 | 1 | 19 | 1 |
| 0.2 NAA | 2 2ip | 40 (9/18/10/2/1) | 26 (7/10/6/2/1) | | | | | 26 | |
| | 2 BAP | 94 (21/51/13/8/1) | 75 (18/43/8/5/1) | 3 | | 34 | 11 | 27 | |
| | 2 KIN | 14 (3/6/3/1/1) | 7 (1/4/1/1/0) | 2 | 1 | 2 | | 2 | |
| No auxin | 2 2ip | 21 (5/7/6/2/1) | 15 (3/5/4/2/1) | | | 2 | | 12 | 1 |
| | 2 BAP | 12 (4/7/1) | 6 (2/3/1) | | | 3 | | 3 | |
| | 2 KIN | 10 (3/6/1) | 4 (2/2/0) | | | | | 4 | |
| Total | | 244 | 163 | 5 (3.1) | 1 (0.6) | 43 (26.4) | 12 (7.4) | 100 (61.3) | 2 (1.2) |

[a] Number of shoots regenerated / subculture.
[b] Number of plants transferred to soil / month.
[c] Number of plants / total number of regenerants in soil.

arm of chromosome 6. One 18S-5.8S-26S rDNA site was observed in the secondary constriction including flanking short chromosomal segments of satellite and terminal regions on the short arm of chromosome 8 (Figure 1B).

At30 had the same 5S rDNA sites as wild-type *A. tuberosum* (Figure 1C), but it differed for the number of 18S-5.8S-26S rDNA sites. The signa1 of 18S-5.8S-26S rDNA was detected in only three copies of chromosome 8 (Figure 1D). On the basis of FISH markers found in chromosomes of wild-type *A. tuberosum* (Figure 2A), At30 lost two chromosomes. The lost chromosomes of At30 were chromosome 7 on which rDNA sites were not detected and chromosome 8 with the 18S-5.8S-26S rDNA site (Figure 2B).

## Discussion

The production of regenerants through tissue culture is increasingly useful for a range of purposes. Their primary purpose is the induction of somaclonal variation. Previous authors have reported a wide range of numerical variations of chromosome among regenerated plants (D'Amato, 1977; Sekerka, 1977). In *A. tuberosum*, Pandey et al. (1992) reported that all the regenerated plantlets had the normal chromosome number. In our study, however, numerical variation was observed among regenerated plantlets. In spite of the reduction in chromosome number during tissue culture, a level of viability similar to that of wild-type *A. tuberosum* was maintained in variants, especially in At30. At30 showed the highest adaptability among aneuploid variants, vigorous growth and genomic stability for two generations. One aim of present study was to consider the possibility of breeding the somaclonal variant as a new crop. Therefore, At30, the new somaclonal variant obtained from this study, can be used as valuable in breeding programs of *A. tuberosum.*

In recent years, DNA-based marker technologies have made a major contribution to detect somaclonal variation, particularly markers based on restriction fragment length polymorphisms (RFLPs) have led to reports of mutation in regenerants (Kidwell and Osborn, 1993; DeVerno et al., 1994). Others have attemped to detect somaclonal variation using randomly amplified polymorphic DNA (RAPD) technology (Munthali et al., 1996; Shoyama et al., 1997) and banding changes have been reported among the somaclones of grass species (Brown et al., 1993). Numerous reports indicate that changes in repetitive DNA are common occurrence in tissue culture (Deumling and Clermont, 1989; Karp et al., 1992; Leitch et al., 1993). Even though changes in chromosome structure of regenerated plants has been shown through meiotic pairing behavior (Dahleen and Eizenga, 1990), only a few studies have documented changes in chromosome number using molecular markers in regenerated plants. Therefore, the application of FISH to regen-

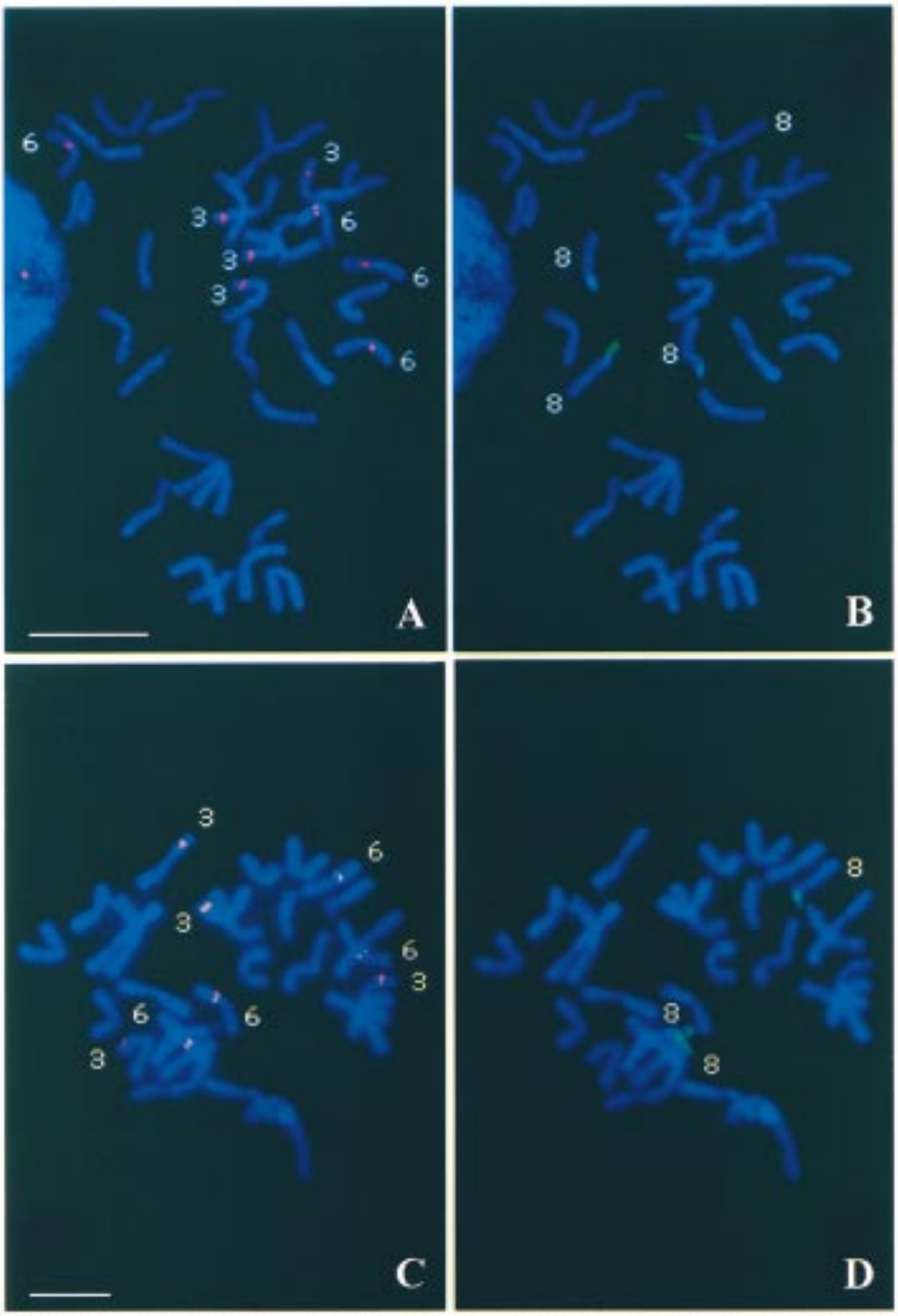


*Figure 1*. FISH patterns using the 5S and 18S-5.8S-26S rDNA probes of wild *A. tuberosum* metaphase chromosome (*A* and *B*) and At30 (*C* and *D*) (bar=10μm). Digoxigenin-labelled 5S rDNA probe detected with antidigoxigenin-rhodamine conjugate (red) (*A* and *C*) and biotin-labelled 18S-5.8S-26S rDNA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

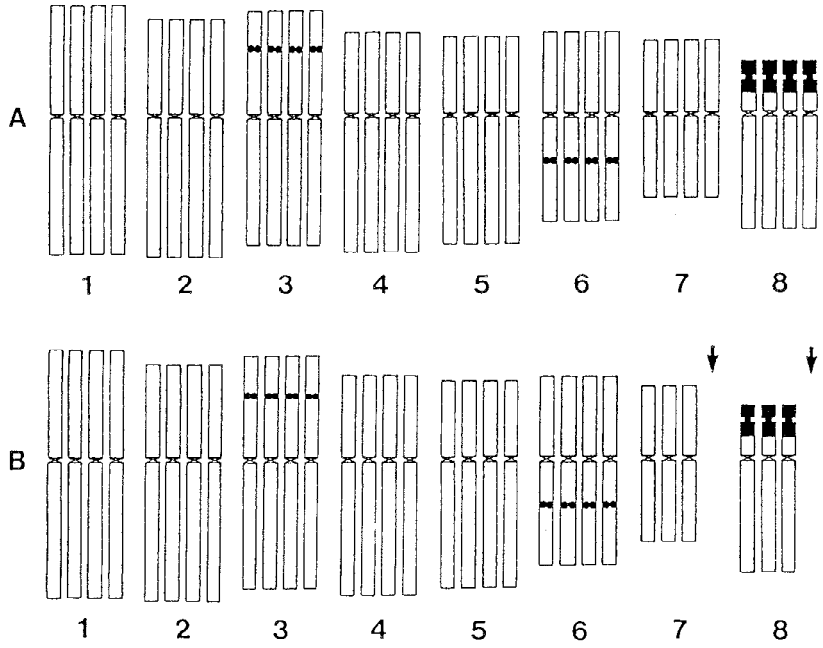


*Figure 2.* Idiogram for chromosomal localization of 5S and 18S-5.8S-26S rDNAs observed by FISH in both wild *A. tuberosum* (A) and At30 (B). Circles indicate 5S rDNA and rectangles indicate 18S-5.8S-26S rDNA loci. The arrows indicate the deleted chromosome which is responsible for the abnormal karyotype.

erants may be a useful tool in identifying and understanding chromosomal changes in the tissue culture process. However, FISH technology has only been used to compare the chromosomal location of rDNAs in the analysis of a callus-derived *Allium* species (Lee et al., 1998). This previous analysis of somaclonal variation using molecular markers tends to demonstrate the numerical doubling of wild-type diploid chromosomes in the tetraploid regenerants. Hence, the application of the FISH technique for the analysis of karyotypic change in aneuploid At30 has allowed the cytologically based physical localization of molecular markers such as 5S and 18S-5.8S-26S rDNA probes. Chromosomal locations of both rDNAs in At30 were correspondent with those of wild *A. tuberosum*, and are independent arrays in chromosome 3, 6 and 8 (Figures 1 and 2) but differed for the number of 18S-5.8S-26S rDNA sites. We found that one chromosome of chromosome 8 carrying 18S-5.8S-26S rDNA site has been lost during the period of tissue culture, but this may not affect growth.

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