Plant Cell Reports (1998) 18: 209–213

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Chromosome analysis by fluorescence in situ hybridization of callus-derived regenerants in *Allium cyaneum* R.

Received: 20 March 1998 / Revision received: 15 June 1998 / Accepted: 8 July 1998

Abstract Investigations were performed to confirm the optimal in vitro culture condition for callus induction and plant regeneration, to observe if somoclonal variation occurs among regenerated plants at the ploidy level and to analyse the chromosomal location of 5S and 18S-26S rRNA gene families using fluorescence in situ hybridization in callus-derived plants of *Allium cyaneum*. Highest callus initiation was achieved with bulb explants cultured on MS medium supplemented with 2,4-D and BAP at 1 mg l^{-1} each. A total of 195 plants was obtained when using MS medium supplemented with 1 mg l^{-1} NAA and 5 mg l^{-1} BAP; about 92% were diploid having 2n = 16; 8% showed a variation in ploidy level. Using digoxigenin-labelled 5S rRNA and biotin-labelled 18S-26S rRNA gene probes, we compared the fluorescence in situ hybridization patterns of autotetraploid plants with the *A. cyaneum* wild type. The 5S rRNA gene sites were detected on the interstitial region in the short arm of chromosome 4 and on the interstitial region in both arms of chromosome 7. The 18S-26S rRNA gene sites were detected on the terminal region of the short arm, including the satellite of chromosome 5, as well as on a part of chromosome B. The chromosomal location of both rRNA genes in regenerated autotetraploid plants corresponded to those of the wild species.

Key words Somaclonal variation · *Allium cyaneum* · Fluorescence in situ hybridization · Autotetraploid regenerants

Communicated by F. Constabel

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Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid · *BAP* 6-benzylamino-purine · *MS* Murashige and Skoog (1962) medium · *NAA* α-naphthalene-acetic acid · *FISH* fluorescence in situ hybridization · *PCR* polymerase chain reaction · *SSC* saline sodium citrate · *KIN* kinetin

Introduction

Allium cyaneum is a plant found in mountainous regions at altitudes above 1000 m. Karyotypic analysis of this species has revealed somatic chromosomes (2n = 16) including one pair of satellite chromosomes plus 0–5 B chromosomes. Some terminal C-band markers were observed on a few chromosomes (Seo et al. 1989).

Somaclonal variation due to karyotypic changes is a widespread phenomenon in plant cell culture, and it might affect plant breeding and propagation (Larkin and Scowcroft 1981; Wenzel 1985). It has been demonstrated for a number of plant species including *Medicago media* (Nagarajan and Walton 1987), *Kallstroemia pubescens* (Seungupta et al. 1987) and *Lilium longiflorum* (Qu et al. 1988). Chromosome doubling is a common type of somaclonal variation and has been observed for *A. sativum* (Novak 1980; Kim and Seo 1991), *A. cepa* (Roy 1980), *A. wakegi* (Seo and Kim 1988), *A. senescens* var 'minor' (Nair and Seo 1992) and *A. victorialis* var 'platyphyllum' (Seo et al. 1995) as well as other crop plants. There has been much interest in developing in vitro technology to facilitate the breeding of commercially valuable *Allium* species (Jones and Mann 1963).

C-banding is a widely applicable method that has facilitated the identification of separate chromosomes in plants. However, the interpretation of a karyotype by means of C-banding patterns is often difficult when only a few terminal C-banded chromosomes are observed. Examination of the chromosomal distribution of 5S and 18S-26S ribosomal RNA genes is useful in identifying the types of genomic changes that might occur during in vitro culture (Maluszynska and Heslop-Harrison 1993). In plants, 5S

and 18S-26S ribosomal RNA genes are present in many hundreds of tandemly repeated units at one or more pairs of loci within the genome. The use of the fluorescence in situ hybridization (FISH) technique allows the visualization of multicopy gene families such as rRNA genes or repeated sequences in plant chromosomes. Ribosomal RNA genes in particular have been extensively studied for physical mapping using FISH in many species (Bergey et al. 1989; Mukai et al. 1990, 1991; Griffor et al. 1991; Ricroch et al. 1992; Castilho and Heslop-Harrison 1995). FISH using these rRNA genes has been reported for *Allium* species, such as *A. sativum* (Hizume et al. 1995), *A. fistulosum* (Irifune et al. 1995), *A. wakegi* (Hizume 1994) and *A. victorialis* var 'platyphyllum' (Seo et al. 1997). Furthermore, a multicolour FISH technique is a useful tool for simultaneous detection of two or more sequences with different colours in the same cells (Ried et al. 1992; Mukai et al. 1993).

The work presented here focussed on the selection of variants from cultured tissues of wild *A. cyaneum* and applied multicolour FISH using 5S and 18S-26S rRNA gene probes to confirm whether the selected tetraploid variants were autotetraploids.

Materials and methods

Callus culture, plantlet regeneration and chromosome counts

Basal bulbs of *Allium cyaneum* R. collected at 1900 m on Mt. Chiri (Korea) were rinsed in tap water, then surface-sterilized by immersing entire bulbs in 70% (v/v) ethanol for 5 min and in 5% (w/v) sodium hypochlorite solution for 20 min with vigorous agitation, followed by washing three times in sterile distilled water. Basal sections included the shoot apex, middle sections included the leaf sheat bases and the upper sections included only leaf blades. To induce callus formation, we placed all the sections or explants on MS (Murashige and Skoog 1962) and B_5 medium (Gamborg et al. 1968) with hormone combinations as described in Table 1. Following inoculation, callus was cultured for 120 days and then subcultured at 60-day intervals on the same medium. During subculture callus pieces were transferred to MS medium supplemented with 1 mg l^{-1} NAA and 5 mg l^{-1} BAP and maintained under continuous illumination (2000 µE m^{-2} s^{-1}) supplied by cool-white fluorescent light for shoot induction. Regenerated shoots (about 5 cm) were transferred to plant growth regulator-free MS basal medium for rooting. All media were solidified with 8 g l^{-1} agar. After about 1 month rooted plantlets were transferred to soil. The ploidy level was ascertained by counting the number of chromosomes in the root tips of the plantlets using the procedure of Seo et al. (1989).

In situ hybridization, microscopy and photography

The pTa71 clone is a 9.4-kb-long DNA sequence derived from rye, *Secale cereale*, and inserted into plasmid pUC18. The probes were labelled with biotin-16-dUTP by the nick translation method according to the manufacturer's protocol (Enzo Diagnostics, Farmingdale, N.Y.). The 320-bp-long 5S rDNA derived from *Allium fistulosum* was labelled with digoxigenin-11-dUTP by the PCR (polymerase chain reaction).

Chromosome preparation and in situ hybridization were carried out using the method of Mukai et al. (1993). On slides, chromosomal DNA was denatured in 70% formamide at 67 °C for 2 min and dehydrated sequentially in 70%, 95% and 100% ethanol at −20 °C for 5 min each. The probe mixture containing 50% formamide, 10% dextran sulphate, 2×SSC, 500 µg/ml salmon sperm DNA, 200 ng/µl 5S rDNA probe labelled with digoxigenin-11-dUTP and 200 ng/µl 18S-26S rDNA probe labelled with biotin-16-dUTP was denatured at 100 °C for 10 min and then kept on ice for 5 min. Ten microliters of the hybridization mixture was dropped onto, each slide, and the slides were covered with coverslips. The hybridization was carried out over more than 6 h at 37 °C in a humid chamber. After hybridization, the coverslips were removed in 2×SSC, and the slides were then washed in 2×SSC for 5 min, 50% formamide for 15 min at 37 °C, 2×SSC for 15 min, 1×SSC for 15 min and 4×SSC for 5 min, which allowed binding of the probe with minimal homology. The slides were covered with 50 µl of anti-digoxigenin-rhodamine conjugate for the digoxigenin-labelled 5S rDNA probe, and 50 µl of avidin-FITC (fluorescein isothiocyanate) conjugate for the biotin-labelled 18S-26S rDNA probe mixture dissolved in 1% BSA/4×SSC as detection buffer, and incubated for 1 h at 37 °C without coverslips. Thereafter, slides were washed in 4×SSC for 10 min, 4×SSC/0.1% Triton X-100 for 10 min, 4×SSC for 10 min and 2×SSC for 5 min at room temperature. Fifteen microliters of a DAPI (4,6-diamidino-2-phenylindole) solution (1 µg/ml) containing Vectashield antifading solution as a counterstain was added onto each slide and overlaid with a coverslip.

The detection of in situ hybridization signals was conducted using a Carl Zeiss Axioplan epifluorescence microscope equipped with filter sets No. 15 (Rhodamine), 09 (FITC) and 02 (DAPI). Photographs were taken on KODAK 400 colour print film.

Table 1 Percentage of callus formation of bulb explants from *Allium cyaneum* on different culture media

Basal medium	Plant growth regular (mg/l)				Percentage of callus initiation (no. of initiated callus per explant)	Callus type
	Auxin		Cytokinin			
	2,4-D	NAA	BAP	KIN		
MS	1				–	
	1		1		67[a] (135/200)	A
	1			1	35 (61/172)	B
	1		1	1	45[a] (56/125)	C
	1	1		1	–	
		1			–	
		1	1		–	
		1		1	–	
B_5	1				29 (40/138)	D
	1		1		22 (26/117)	E
	1		2		19 (27/143)	F
	1		1	1	–	
	1	1		1	24 (29/122)	G

[a] Simultaneous formation of callus and shoot

Results

Tissue culture

Seven types of calli were obtained using seven media which differed in basal composition and plant growth regulators (Table 1). Maximum frequency of callus formation was achieved on MS medium supplemented with 2,4-D and BAP at 1 mg l^{-1} each. While calli did not appear until 120 days after inoculation in callus types A and C, callus type B showed continuous growth beginning at 30 days after inoculation of the explants. Among MS basal media,

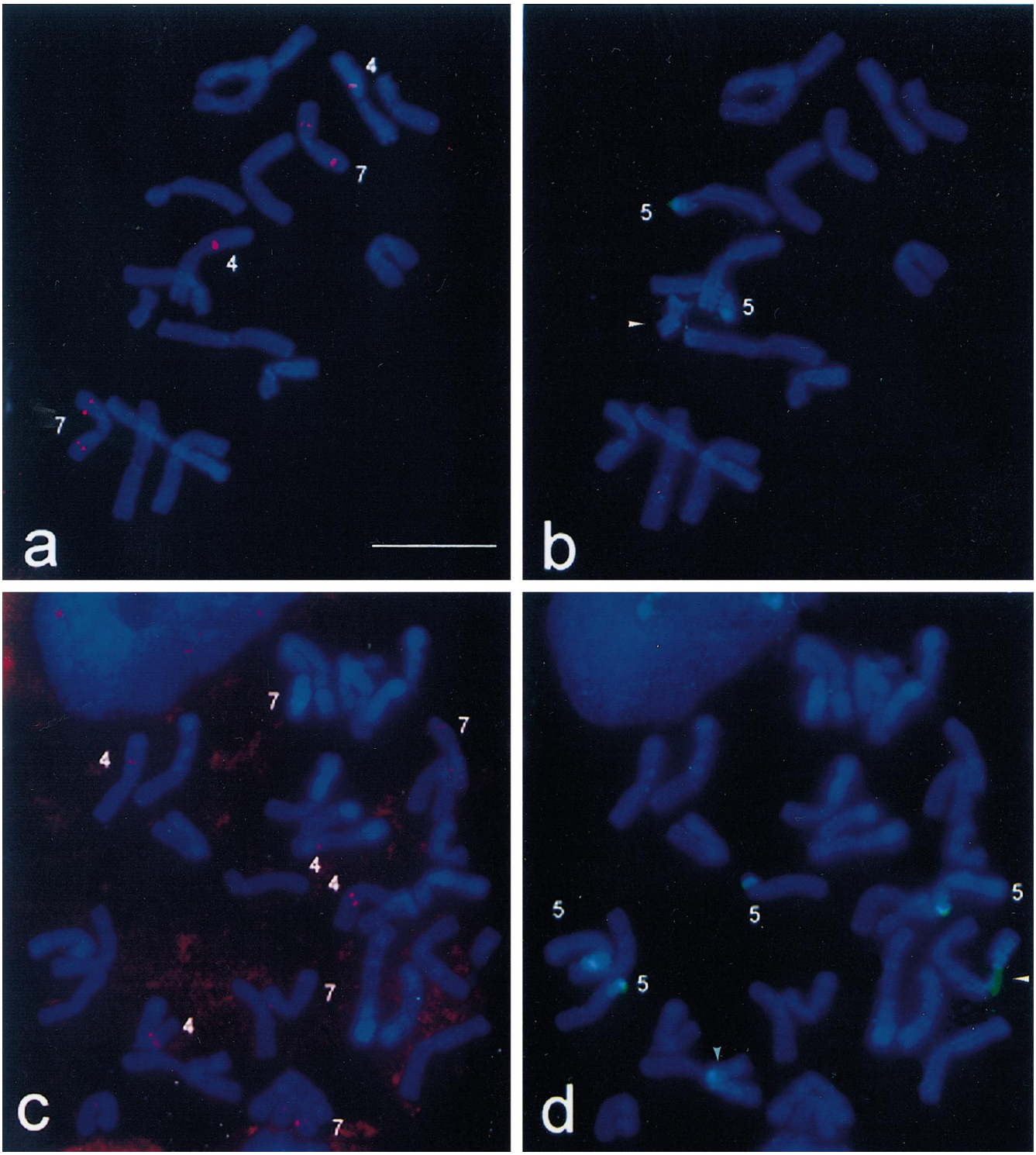

Fig. 1 a–d FISH of 5S and 18S-26S rRNA genes in metaphase plates of wild type (**a, b**) and tetraploid regenerant (**c, d**). Digoxigenin-labelled 5S rRNA gene probe was detected with rhodamine (**a, c**) and biotin-labelled 18S-26S rRNA gene probe with FITC (**b, d**). *Numbers* indicate corresponding chromosomes showing rRNA gene signals. *Arrowheads* indicate B chromosomes. *Bar:* 10 μm

callus type B was characterized by a yellowish round pattern. In types A and C, callus and regenerated shoots grew simultaneously. On B_5 medium, callus initiation was below 30%.

Calli showing a greenish colour were forming shoot primordia directly when subcultured on shoot induction medium. More than 95% of the calli developed shoots via organogenesis on shoot induction medium supplemented

Table 2 Number of plant regenerants of *Allium cyaneum* and ploidy level checked with primary root tips of plantlets based on callus type

Callus type	Number of regenerants	Number of			
		Diploids	Hyper-diploids	Mixo-ploids	Tetra-ploids
A	66	61	2	1	2
C	92	83	2	4	3
D	18	17	–	–	1
F	19	18	–	–	1
Total	195	179	4	5	7

with 1 mg l^{-1} NAA and 5 mg l^{-1} BAP. Shoot buds were produced from the surface of the calli grown on media A, C, D and F. In callus types B, E and G, no shoot regeneration was observed.

Shoots subsequently produced roots on plant growth regulator-free MS medium and developed into plantlets. A total of 195 plantlets was transplanted to soil in pots, and these developed into phenotypically normal plants within 6 weeks after being transferred into a vermiculite perlite mixture and watered daily with Hoagland's solution.

Cytological analysis of the regenerated plants

Among the 195 regenerants, 179 (92%) were diploid (2n = 16) and 16 were variants including 4 hyperdiploids, 5 mixoploids, and 7 tetraploids (Table 2). The percentage of hyperdiploids and mixoploids decreased and became fixed diploids or tetraploids, thus totalling 12 tetraploid individuals (data not shown).

Fluorescence in situ hybridization (FISH)

A. cyaneum has one pair of submedian chromosomes having a satellite and seven pairs of median chromosomes, with 0–5 B chromosomes (Fig. 1). Digoxigenin-11-dUTP labelled 5S rRNA genes were detected on the interstitial region in the long arm of chromosome 4 and on interstitial regions in the short and long arms of chromosome 7. The signals of the biotin-16-dUTP-labelled 18S-26S rRNA gene corresponded to the secondary constriction including flanking short chromosomal segments of satellites and the short arm of chromosome 5. The 18S-26S rRNA gene signal was strong on the terminal region of the B chromosomes. Although a variable number (0–5) of B chromosomes appeared in the root-tip cells, the 18S-26S rRNA gene signals were detected on the terminal region in all B chromosomes observed. Figure 2 shows the chromosome and interpretative drawings having rRNA gene loci. Chromosomal locations of both rRNA genes in regenerated tetraploids corresponded with those of wild *A. cyaneum*.

Discussion

Technology to regenerate plants from callus cultures of *Allium cyaneum* permits new approaches of breeding and genetic studies, such as the recovery of useful somaclonal variants or of traits selected at the cell level (Phillips and Hubstenberger 1987).

FISH provides an efficient method for identifying the chromosome composition of regenerants by demonstrating the number and physical location of 5S and 18S-26S rRNA gene loci. The tetraploid regenerants showing numerical doubling of wild-type diploid chromosomes corresponded with the physical localization of rRNA genes of diploid species. The results of FISH suggest that tetraploid regenerants originated from the exact doubling of normal diploids. In situ hybridization with the 18S-26S rRNA genes on *Allium* chromosomes preferentially gave major signals in the satellite and extra B chromosomes. At one time it was believed that there were no genes on B chromosomes. This meant that the activation of the NOR (nucleolus organizer region) was suppressed easily on B chromosomes (Claudio 1986). In recent years, however, there have been some reports of genes on B chromosomes in *Secale cereale* (Cuadrado and Jouve 1994) and *Festuca* species (Thomas et al. 1997), and in the overwhelming majority of these cases they were rRNA genes (Jones 1995). From the results shown in Fig. 2 it is clear that activation of NORs are represented on B chromosomes. Alvarez et al. (1991) reported that B chromosomes can control both the level of chromosome pairing and chiasma formation. They suggested that the B chromosomes seem to affect homologous chromosome association by promoting meiotic A-chromosome pairing. Timmis et al. (1975) studied the reassociation kinetics of DNA in plants carrying 0–6 Bs and demonstrated for the first time the identity of DNA of A and B chromosomes. Differences in sequence organiza-

Fig. 2 Photomicroscopic chromosomes and interpretative drawings indicating rRNA sites in *Allium cyaneum*. *Green* indicates 18S-26S rRNA gene site, and *red* indicates 5S rRNA gene sites

tion between As and Bs in rye was also shown by Tsujimoto and Niwa (1992). These authors did not show any rDNA signals in *Allium* species containing extra B chromosomes in the wild population in Korea such as *A. cyaneum* var 'deltoides', *A. thunbergii* and *A. deltoide-fistulosum* (data not shown).

The results of our investigation indicate the potential for genetic manipulation of wild-type *Allium* species using tissue culture. This is the first time that using the FISH technique autotetraploid variants obtained through callus culture were shown to have been arrived at by chromosome doubling.

Acknowledgements This study was supported by the Basic Science Research Institute Program, Ministry of Education, Korea, 1996, Project No. BSRI 96-4404.

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