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Karyological Variation of Callus-derived Regenerants in *Allium victorialis* var. *platyphyllum*

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The calli obtained from the bulbs of *A. victorialis* var. *platyphyllum* on MS basal medium containing 2 mg/L 2,4-D and 1 mg/L BA. Plants from calli were regenerated on MS basal media supplemented with combinations of NAA and four kinds of cytokinin, BA, zeatin, kinetin and 2iP, and with only each cytokinin. The good response of shoot regeneration was observed in the media with combinations of NAA and BA or zeatin, and with only BA or zeatin. Shoot regenerating response in the medium with combinations of NAA and BA or zeatin was about two times higher than that in the medium with only BA or zeatin. From the karyotypic analysis of the regenerated plants, abnormal diploids, aneuploids and mixoploid with structural aberrations were investigated. The somaclonal variants (AV 16-04, AV 13-03) were shown the considerable differences from normal diploid regenerant (AV 18-03) in the external morphology.

Keywords: karyotypic analysis, diploid, aneuploid, mixoploid, structural aberrations

Somaclonal variation is a widespread phenomenon and a major importance for those intending to apply tissue culture in plant breeding and propagation. As to breeding, the use of somaclonal variation as novel source of variability for plant improvement has been suggested (Larkin and Scowcroft, 1981; Wenzel, 1985). The cytological characteristics commonly noticed in somaclonal variation are structural or numerical variations of chromosome such as polyploidy, aneuploidy, chromosome rearrangements (Lee and Philips, 1987; Nagarajan and Walton, 1987; Seungupta *et al.*, 1987; Qu *et al.*, 1988). Somaclonal variation has been recorded for most species capable of plant regeneration from tissue culture, and variation has been detected in terms of phenotypic and genotypic characteristics (Müller *et al.*, 1990; Linacero and Vazquez, 1992; Nehra *et al.*, 1992). Many chromosome features help to understand the genic homologies, structural variation and numerical differences. Chromosomal reorganisation at various levels may result in numerical changes and errors of mitosis and meiosis are however more serious and leads to the formation of polyploid and aneuploid cells or chromosome mosaicism. Chromosomal changes are a clear indication of somaclonal variation (Lee and Phillips, 1988). Heritable characteristics of somaclones can be studied through genetic and cytological analysis.

Studies on tissue culture of the genus *Allium* have been reported in *A. sativum* (Kim and Seo, 1991), *A. cepa* (Yamane, 1975), *A. wakegi* (Seo and Kim, 1988), and *A. senescens* var. *minor* (Nair and Seo, 1992). *A. victorialis* var. *platyphyllum*, a perennial wild plant of *Lilium* family, grows naturally in Odaesan, Jirisan and Seolagsan Mt., and in Ulreung Is. Kim *et al.* (1996) reported a method for *in vitro* development of plantlets via somatic embryogenesis and organogenesis in *A. victorialis*. Seo *et al.* (1995) reported the karyological analysis in the callus cells of *A. victorialis*. The aim of this work was to obtain the chromosomal variants among regenerated plants, and to compare the external features with normal plants.

MATERIALS AND METHODS

The bulbs of *Allium victorialis* var. *platyphyllum* collected from natural populations of Is. Ulreung were surface sterilized immersing in 70% ethanol for 5 min and 7% sodium hypochlorite for 20 min and followed by washing three times in sterile distilled water. The callus initiation media used were

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BDS media (Dunstan and Short, 1977) supplemented with 2 mg/L 2,4-D and 1 mg/L BA. Callus was initiated under continuous illumination (3, 000 lux) by cool white fluorescent light at 25±1°C and 60±5% relative humidity, and then transferred to dark condition for callus growth. The induced callus was initially subcultured for 210 days after inoculation, and subsequently subcultured in 30 days interval on the same medium. At the same time, actively dividing callus was transferred to MS (Murashige and Skoog, 1962) media supplemented with combinations of NAA and four kinds of cytokinin, BA, zeatin, kinetin and 2iP, and with only each cytokinin for shoot regeneration as shown in Table 1. For the root formation, shoots about 3-4 cm long were isolated and transferred to the same basal medium without any growth hormones. After one month, the rooted plantlets were transferred to paper cups containing sterilized vermiculite soaked with 100 ml Knop solution, protected by vinyl bags to maintain humidity and kept at room temperature (28-30°C) under 16 hours photoperiod.

For karyological analysis, actively growing root tips were pretreated in cold water for 21 hours at 4°C, fixed in acetic ethanol (1 : 3) overnight and stored in 70% ethanol. Root-tips were hydrolyzed for 20 sec at 60°C in 1 N HCl and then preparations were made with aceto-orcein by squash method. Giemsa C-banding methods were performed according to Nair and Seo (1995).

RESULTS

The period needed for callus initiation and plant regeneration was relatively long compared to other *Allium* species, such as *A. wakegi, A. sativum, A. fistulosum* and *A. senescense* var. *minor.* Callus initiation on basal bulb explants was visible after 60 days of culture in BDS basal medium containing 2 mg/L 2,4-D and 1 mg/L BA. After 210 days, callus was sufficiently multiplicated for subculture (Fig. 1a). The induced calli were inoculated on MS media with hormone combinations as given in Table 1. After 5 to 7 weeks the basal portion of the callus formed primordia which developed into green healthy shoot buds under continuous illumination (Fig. 1b). The roots were developed directly from callus in MS basal medium supplemented with kinetin alone (Fig. 1c, Table 1). The mode of regeneration from callus was dependent on the hormone used (Fig. 1d). Maximum number of shoots was obtained from the callus maintained in MS medium with 0.2 mg/L NAA and 2 mg/L zeatin. Shoot regeneration was not occurred on four different media supplemented with combination of NAA and kinetin or 2iP, and with only kinetin or 2iP (Table 1). The roots appeared within two weeks after the shoots were transplanted to MS basal medium without hormones.

Somatic chromosomes of all clones used in the present study were 2n=16 which contain one pair of nucleolar chromosomes (chromosome 4) (Fig. 1e).

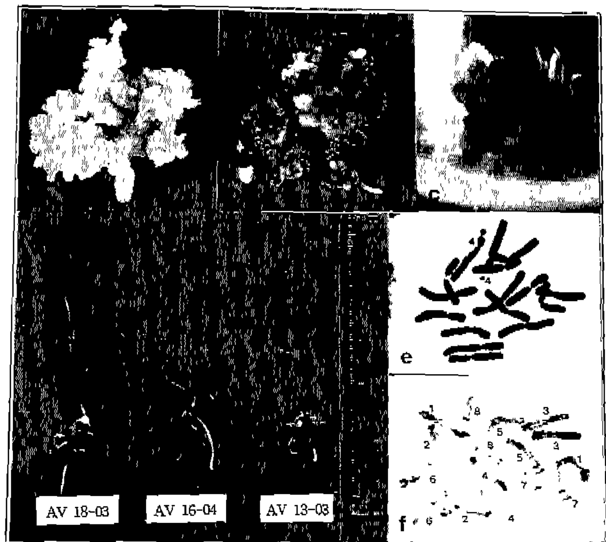

Fig. 1. Regeneration of plants via callus from basal bulb segments of *A. victorialis.* a, callus formed on BDS medium supplemented with 2 mg/L 2,4-D and 1 mg/L BA; b, shoot primordia formation after 240 days; c, direct root regeneration from callus; d, regenerated plantlets; e, somatic metaphase chromosomes (2n=16) in a normal diploid plant; f, C-banded metaphase chromosomes. "sp" indicates shoot primordia and "r" indicates directly regenerated root from callus.

Table 1. Combination and concentration of hormones used for shoot regeneration from callus

Plant growth regulators (mg/L)					% regenerating response
Auxin	Cytokinin				
NAA	BA	zeatin	kinetin	2iP	
0.2	2				46(16/35)
0.2		2			77(23/30)
0.2			2		-
0.2				2	-
	2				23(5/22)
		2			35(6/17)
			2		a
				2	-

Numbers in parenthesis indicate total regenerated shoots/number of callus.
a: root regeneration only

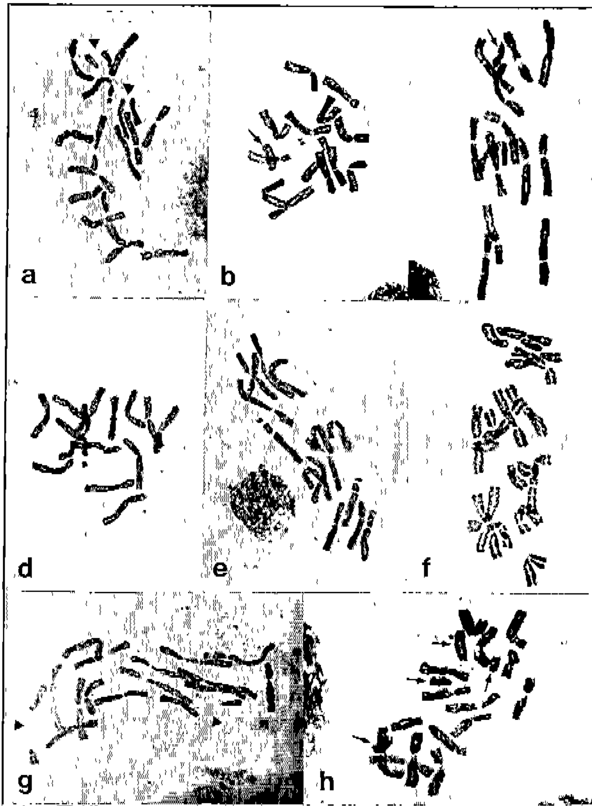

Fig. 2. Aberrations in chromosome number and structure found in regenerants. a, abnormal diploid with centromere breakage; b and c, aneuploids with one-arm deleted chromosome; d, e and f, aneuploids; g and h, aneuploids with structural changes. ▶ indicates centromere breakage. → indicates one arm of chromosome.

The sixteen chromosomes were arranged into 8 pairs by the combination of characters such as total length, arm ratios and satellite chromosome according to Levan *et al.* (1964). C-banding pattern in the chromosome preparation from root tips of about 20 bulbs showed almost identical pattern except several minor bands (Fig. 1f). A pair of nucleolar chromosomes showed distinct bands at secondary constrictions and satellites. All the terminal regions of the short arms appeared to be darkly stained, but terminal regions of the long arms were weakly stained. In the intercalary regions of both arms of all the chromosomes, every homologous chromsomes showed a few symmetrical minor bands which is enough for the identification of each chromosome.

Karyological analysis of the regenerated plants from orcein-stained metaphases revealed that 97.8% of the regenerated plants showed normal karyotype as shown in Fig. 1e. The highest frequency of chromosomal aberration among regenerants was aneuploid (Fig. 2a). The karyotypic variants were classified into diploid, hypodiploid, mixoploid and hyperdiploid for chromosome number. Numerical and structural variations of chromosome in the regenerated plants were summarized in Table 2 and shown in Fig. 2. The hypodiploid having the one arm deleted chromosome following centromere breakage were also observed (Fig. 2b and c). The aneuploids with deletion or addition of some chromosomes were shown in Fig. 2d, e and f. In Fig. 2g and h, the hyperdiploid containing addition of entire chromosome or one arm-deleted chromosome by centromere breakage were also observed.

Table 2. Normal regenerants and six variants showing the altered chromosome composition

Variant	Somatic chromosome number (2n)	Remarks
Normal	diploid (16)	
AV 10-01	〃	centromere breakage
AV 17-02	〃	dicentric chromosome
AV 16-04	hypodiploid (14)	loss of two chromosomes and one arm deletion
AV 13-03	mixoploid (15 and 16)	
AV 17-09	hyperdiploid (17)	addition of a chromosome

Variant AV 16-04 was aneuploid which is showed the somatic chromosomes of 2n=14 containing a chromosome with one arm loss (Fig. 2b, Table 2). The regenerant AV 13-03 was a mixoploid that chromosome number were mixed 2n=15 and 16 (Table 2). As shown in Fig. 1d, this variant was shown the considerable difference in comparison with normal diploid regenerant AV 18-03. Structural rearrangements as translocation or inversion could not be identified by orcein-staining method. But dicentric chromosome, centromere breakage and chromosome fragment were frequently observed from the regenerants.

DISCUSSION

It is evident that numerical and structural variability of chromosomes are occurred among callus cells and regenerants under a wide range of culture environments. But the chromosomal studies on *in vitro* cultured callus and callus-derived regenerated plants are seldom reported so far. This paper describes the karyotypic variation from the root-tips of callus-derived regenerated plants.

The period for callus initiation and plant re-

generation was relatively longer than that of *A. fistulosum* (Kim and Seo, 1987), *A. wakegi* (Seo and Kim, 1988), *A. sativum* (Kim and Seo, 1991) and *A. senescense* var. *minor* (Nair and Seo, 1992). From the culture of *A. victorialis* var. *platyphyllum*, numerical variations of chromosome among the callus cells were correlated with the mitotic abnormalities (Seo *et al.*, 1995). They reported that the callus induction was most suitable on BDS medium, while direct shooting from explants was most suitable on MS medium. Therefore, the medium for shoot regeneration from callus was selected the MS basal medium. Good response of shoot regeneration was on MS medium supplemented with NAA and zeatin. The effect of shoot regeneration in the medium supplemented with only cytokinin was not better that with combination of NAA and cytokinin (Table 1). Among the cytokinins with NAA and zeatin or BA worked well for shoot induction. In case of only cytokinin addition, BA or zeatin was shown some response for shooting. Coleman and Ernst (1990) suggested that zeatin was more effective than BA for shoot proliferation from axillary bud explants of *Populus* spp. Kim *et al.* (1996) reported that shoots formed directly when bulb explants of *A. victorialis* were cultured on MS medium containing 0.2 mg/L NAA and 2.0 mg/L zeatin. They were also suggested that the effect of zeatin was two times better than that of BA although direct shoots were obtained from bulb explants. Similar results were reported from root culture of *Populus alba* x *P. grandidentata* (Son and Hall, 1990). In the present study, shoot regenerating respone in the media supplemented with combinations of NAA and BA or zeatin was about from 2.0 to 2.2 times higher than that in the media containing BA or zeatin alone. Thus, it is suggested that the addition of auxin in the culture medium become a necessary factor in *in vitro* culture for shoot regenerartion in *A. victorialis*.

Although the ploidy of chromosome was not observed, various aneuploid and mixoploid regenerants were investigated in this study. Relationship between different hormone combinated medium and karyological variability was not observed in this study. Aneuploids and mixoploid regenerants with structural changes were sometimes appeared in some regenerated plants. Sacristan (1971) observed the various ploidies and structural changes of chromosome in *in vitro* cultured callus of *Crepis capillaris* and analyzed some karyotypic changes through general staining method. Some authors reported that the number variations were occurred due to simple addition or loss of chromosomes through non-disjunction during mitosis and the structure variations may be due to chromsome breakage with or without translocation, inversion and reunion (Sacristan, 1971; Cummings *et al.*, 1976). The unique C-banding patterns of each chromsome as chromosomal marker in *A. victorialis* var. *platypyllum* will be helpful for analysis of the chromosomal variation originated from *in vitro* culture.

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