Note

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Cytological and RAPD (Random Amplified Polymorphic DNA) Analyses of Somaclonal Variation in Easter Lily (*Lilium longiflorum* Thunb.)

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Abstract

Long-term maintenance of plant cells in tissue culture is known to induce somaclonal variation in regenerated plants. Here we examined somaclonal variation in regenerated plants of Easter lily (*Lilium longiflorum* Thunb.) using cytological and molecular analyses. Forty nine 10-mer and 12-mer arbitrary primers were used to amplify DNAs extracted from sixty plants regenerated after more than two years of callus culture originally derived from bulb tissues. Sixteen primers were found to produce 34 polymorphic bands in the regenerated plants, including both loss of parental bands and appearance of novel, non-parental bands. The presence of polymorphic fragments among the regenerants indicates that genomic alterations occurred during the long-term culture of the cells. Although no variation was observed from karyotype analysis, the present study revealed the high frequency of the occurrence of somaclonal mutations at the DNA level during the callus culture of Easter lily.

Plant tissue culture has enormous potentials as a tool in plant breeding programs. It was often considered as an efficient method for asexual rapid propagation compared with conventional methods. Therefore, it is necessary that all plants regenerated from tissue cultures are identical with each other and their parental form. However, it has been reported for many plants that phenotypic or genotypic variations occurred when cultured for a long period of time.

Somaclonal variation has been found to be associated with various types of genetical changes, such as numerical and structural aberrations of chromosomes (Orton, 1983; Swedlund and Vasil, 1985), deamplification and amplification of genes, single gene mutations, activation of transposable elements (Peschke and Phillips, 1991), and hypo- and hypermethylation of DNA (Scowcroft, 1985). Raja *et al.* (1992) mentioned that chromosomal rearrangements and/or numerical variation of chromosomes are still considered to play a major role in inducing somaclonal variation. Karyological analysis can reveal significant chromosomal changes such as alteration of ploidy level as well as structural rearrangements (Brown *et al.*, 1993). However, karyological analysis cannot reveal alterations in the individual genes. RAPD (Random Amplified Polymorphic DNA) analysis is one of the methods that can partly overcome this limitation and be easily adopted to detect the variations in plant material at a DNA level during all culture and growth stages. The application of RAPD on the analysis of somaclonal variation has been conducted in rice (Godwin *et al.*, 1997), peach (Hashmi *et al.*, 1997), *Querqus suber* L. (Gallego *et al.*, 1997), and *Hordeum* spp. (De Bustos *et al.*, 1998). However, these analyses did not clarify the possible association of changes at a molecular level to those at a chromosomal level.

In the genus *Lilium*, RAPD analysis has been applied to determine the relationship between and within the species (Yamagishi, 1995). However, no study has so far been reported on the application of RAPD to examine somaclonal variations in *Lilium*. The present paper reports the karyotype and RAPD analyses of regenerants of Easter lily after a long-term callus culture.

Calli of *Lilium longiflorum* 'Georgia' were induced from scale segments of *in vitro*-grown bulbs on 0.2% Gelrite (Scott Laboratories)-solidified MS medium (Murashige and Skoog, 1962) supple-

mented with 1 mg l^{-1} picloram and 3% sucrose, and were maintained on the same medium by subculturing every two weeks for more than two years. For obtaining regenerated plants, calli were transferred onto MS medium supplemented with 0.1 mg l^{-1} NAA instead of picloram. Sixty regenerated plants growing on this medium were used for subsequent analyses.

For cytological analysis, root tips of the regenerated plants were collected, pretreated with ice-cold water for 24 h and placed in Farmer's fixative (3:1 ethanol:acetic acid) overnight. Chromosome preparations of metaphase cells were made by treating root tips with an enzyme solution containing 1% Cellulase Onozuka RS (Yakult Honsha Co., Ltd.), 0.5% Hemicellulase (Sigma) and 1% Pectolyase Y-23 (Seishin Pharm. Co., Ltd.) for one hour at 37 ℃. Chromosomes were stained with 1% aceto-carmine and observed under a light microscope.

Cytological analysis indicated that there was no variation at a chromosomal level both in the chromosome number and structural rearrangement (**Fig. 1**). All of the sixty regenerants analyzed were found to have the same karyotype as that of the parental plant with 24 chromosomes, *i.e.*, two pairs of sub-metacentric (A, B), seven pairs of sub-telocentric (C, D, E, F, G, H, I), and three pairs of acrocentric chromosomes (J, K, L). A pair of chromosome C had the secondary constrictions on the long arms. Although karyotype instabilities in cultured tissues have been reported for many plant species (Orton, 1983), the present study showed that karyotype instability in the regenerants of *L. longiflorum* did not occur. A high degree of chromosomal stability in *L. longiflorum* plants regenerated from the cells which had been subcultured *in vitro* for a long period was previously reported by Sheridan (1975).

Ploidy level of the regenerated plants was tested by flow cytometry following Supaibulwatana and Mii (1998). Leaves of the regenerated plants were chopped and stained with DAPI solution [0.1% Triton-X100 and 4 μg ml^{-1} 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) in 10 mM Tris-HCL, pH 7.5]. The cell suspension was passed through a nylon mesh (40 μm) and analyzed for DNA content using a flow cytometer (Partec CA II®, Germany). In our previous study using flow cytometry, friable calli in suspension culture of *L. longiflorum* showed tetraploidy (Supaibulwatana and Mii, 1998). However, in the present study, the regenerants also did not show any polyploidy in the flow cytometry analysis (**Fig. 2**).

In order to investigate somaclonal variation at the molecular level, DNAs were extracted from fresh leaves of the regenerated plants according to the

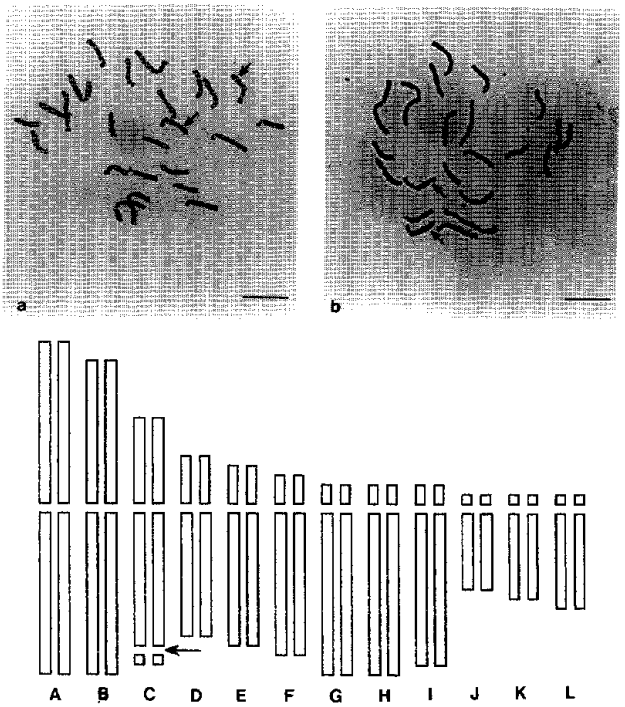

Fig. 1 Somatic metaphase chromosomes of the parental plant (a) and one of the regenerated plants (b). Both showed the same number ($2n$=24) and karyotype (c). Arrows show secondary constrictions. Bar indicates 20 μm

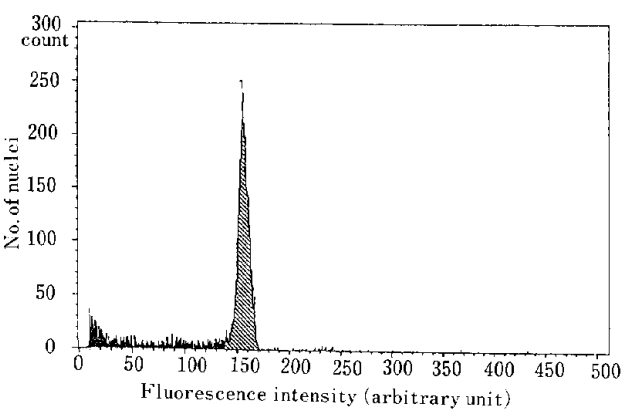

Fig. 2 A representative histogram of flow cytometric analysis of the regenerated plants comparable to the parental plant.

procedures described by Dellaporta *et al.* (1983). The amplification of DNA was performed using Mini Cycle PTC-150 (MJ Research, Inc.) at denaturing temperature of 94 ℃, annealing temperature of 35 ℃ and extension temperature of 72 ℃. The PCR program consisted of 1 cycle of 94 ℃ for 2 min, 35 ℃ for 1 min and 72 ℃ for 2 min; 40 cycles of 94 ℃ for 30 sec, 35 ℃ for 30 sec and 72 ℃ for 2 min; and finally 1 cycle of 94 ℃ for 30 sec, 35 ℃ for 30 sec and 72 ℃ for 10 min before maintaining at 5 ℃. Forty-nine primers were used to explore the polymorphic bands. The decamer primers (OP Primers) were purchased from Operon Technologies Inc. and

Table 1. Primers used and the number of polymorphic bands detected in the regenerated plants.

Primers	Sequence 5′-3′	No. of polymorphic bands	No. of parental bands	No. of novel bands
10-mer:				
OPC-01	TTCGAGCCAG	1	1	–
OPC-03	GGGGGTCTTT	2	1	1
OPC-04	CCGCATCTAC	3	2	1
OPC-05	GATGACCGCC	2	1	1
OPC-06	GAACGGACTC	1	1	–
OPC-07	GTCCCGACGA	3	2	1
OPD-03	GTCGCCGTCA	3	2	1
OPD-06	ACCTGACCGG	2	–	2
12-mer:				
CMN-A04	GCCCCGTTAGCA	2	2	–
CMN-A08	TTCGGACGAATA	2	–	2
CMN-A12	CTCCTGCTGTTG	1	–	1
CMN-A34	CCTGCGGGAGGA	1	1	–
CMN-A45	TGGCCTCTTGGA	2	2	–
CMN-A54	AAGGCGTGTTTA	4	2	2
CMN-A65	CTTGAGCGTATT	3	2	1
CMN-A68	ACTTTCGATCCA	2	2	–
Total		34	21	13

the 12-mer ones (Common's Primers) were purchased from Bex Inc., Tokyo. The amplification products were electrophoresed on a 1.5% gel (Takara LO3) in TAE buffer (40 mM Tris-acetate and 1 mM EDTA) at a constant 100 V. After staining in a 2 μg ml^{-1} ethidium bromide solution, the gels were photographed on a UV transilluminator. The polymorphic bands were scored for the presence or absence of the amplification products. The primers which produced polymorphic fragments were tested repeatedly in order to ensure reproducibility of the polymorphic fragments.

Among 49 primers tested in RAPD analyses, 16 were found to produce polymorphic fragments. The number of bands per primer ranged from 1 to 4, and a total of 34 polymorphic fragments were generated (**Table 1**). Among the 34 polymorphic fragments, 13 (38%) were found to be novel ones which were not observed in the parental plant.

RAPD techniques can potentially detect single base mutations or deletions at the level of the primer target and also insertions and deletions within the amplified fragments (Williams *et al.*, 1990). In the present study, polymorphisms observed among the 60 regenerated plants could be identified by the differences in the fragment sizes and presence or absence of the fragments (**Fig. 3**). The present results indicate that some mutations such as deletion or insertion might occur in the amplified regions

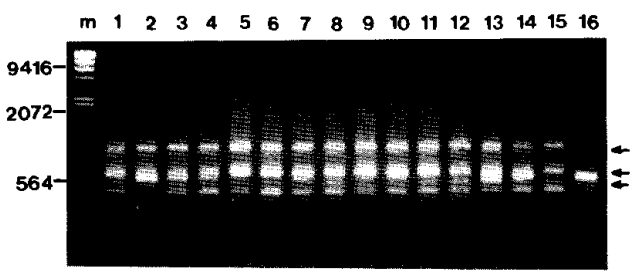

Fig. 3. Representative RAPD profiles showing polymorphism among regenerated plants (Lane 2-16) and the parental plant (Lane 1) of *L. longiflorum*, amplified using OPC-07. Arrows show polymorphic bands. Numerical indicate base pair. m: size marker

and/or that base changes might induce the alteration of primer binding sites.

In rice, it has been reported that alteration of repeated DNA sequences induced genetic variation during the callus culture phase (Zheng *et al.*, 1987). As Sentry and Smyth (1985) identified a family of dispersed repeat sequences longer than 7 kbp in *L. henryi*, it is possible that *L. longiflorum* may also contain abundant repeated sequences in the genome, and that the changes in repeated sequences might occur in the regenerants of *L. longiflorum*. On the other hand, behavior of transposable elements could provide an explanation for the genetic changes among somaclones of plants. A tissue culture envi-

ronment has been reported to be condusive for transposon-induced changes in a number of species such as maize (Peschke *et al.*, 1991). Joseph *et al.* (1990) found the LTR retrotransposon *del*1 which is abundant in *L. longiflorum* and *L. formosanum*. Therefore, such retrotransposons may induce genetic changes in the regenerants of *L. longiflorum*.

In the present study, phenotypic variation of the regenerants was not examined because of their immature status. Therefore, further investigations are needed to clarify the relationship between the variations at the molecular level and those at the phenotypic level.

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Erratum

T. Ikeda, H. Nonami, T. Fukuyama, Y. Hashimoto, **Water potential associated with cell elongation and cell division of tissue-cultured carnation plants,** *Plant Biotechnology*, **16**, 115-121 (1999).

The following 10 typographical errors should be corrected:

1) The scientific name of carnation plants printed on the 3rd line of the **Abstract** on Page 115 as "(*Dianthus caryophyllus L.*)" should be read as "(*Dianthus caryophyllus* L.)", *i.e.*, "L." should not be italic.

2) The unit printed on the 2nd line above Equation 1 in the left column of Page 116 as ": s-1)" should be read as ": s^{-1})", *i.e.*, " − 1" should be superscript.

3) The sentence, "When G is plotted as a function of (p, ...", on the 4th line below Equation 2 in the left column of Page 116 should become "When G is plotted as a function of Ψ_P, ...", *i.e.*, the abbreviation for turgor (Ψ_P) was printed inaccurately.

4) On line 40 of the left column on Page 116, which is 5 lines above Equation 3, the sentence, "..., Eqs. I and 2 can be ...," should be read as "..., Eqs. 1 and 2 can be ...," *i.e.*, "1" was incorrectly typeset as " I ".

5) On the left column on Page 116, the equation 3 was printed inaccurately as

$$G = \frac{ml}{m+L}(\Psi_0 + \Psi_s - Y) \qquad (3)$$

The entire equation with the correction is printed below.

$$G = \frac{mL}{m+L}(\Psi_0 - \Psi_s - Y) \qquad (3)$$

where "l" should be typed as "L", and "+" should become " − " before Ψ_s.

6) In the legend of **Fig. 2** on Page 118, the abbreviation for osmotic potential was printed inaccurately as "osmotic potentials of the elongation zone (Ψ_w^E; ▲) (A)". It should read: "osmotic potentials of the elongation zone (Ψ_S^E; ▲) (A),...", *i.e.*, the subscript "w" should become the subscript "s".

7) On the left column on Page 119, in the paragraph of "*3.4 Factors Controlling Cell Elongation*", the last equation below the sentence, "By using the above approximation, Eq. 3 can be modified as follows;" was printed inaccurately as

$$F = \frac{mL}{m+L}(\Psi_0 - \Psi_s - Y) = \frac{mL}{m+L}\{(\Psi_0 - \Psi_W)(\Psi_p - Y)\} \approx L(\Psi_0 - \Psi_W)$$

The entire equation with the correction is printed below.

$$G = \frac{mL}{m+L}(\Psi_0 - \Psi_s - Y) = \frac{mL}{m+L}\{(\Psi_0 - \Psi_W) + (\Psi_p - Y)\} \approx L(\Psi_0 - \Psi_W)$$

where "F" must be typed as "G", and "+" must be inserted between the parentheses following the second equal sign.

8) In the **References**, the title of the paper written by Morgan (1984) was incorrect. It should be read;

Morgan, J.M., 1984. Osmoregulation and water stress in higher plants. Annu. Rev. Plant Physiol., **35**: 299-319.

9) In the **References**, the title of the paper written by Kozai and Iwanami (1988) was incorrect. It should be read;

Kozai, T., Iwanami, Y., 1988. Effects of CO_2 enrichment and sucrose concentration under high photon fluxes on plantlet growth of carnation (*Dianthus caryophyllus* L.) in tissue culture during the preparation stage. J. Japan. Soc. Hort. Sci., **57**: 279 − 288.

10) In the reference of Wainwright and Harwood (1985), the scientific name of cyclamen plants printed on the 32nd line in the right column on Page 121 as "*Cyclamen* persicum Mill." should be read as "*Cyclamen persicum* Mill.", *i.e.*, "persicum" should be italic.