

Production of Doubled Haploid Plants Through Colchicine Treatment of Anther-derived Haploid Calli in the Asiatic Hybrid Lily 'Connecticut King'

Dong-Sheng Han, Yoshiji Niimi* and Masaru Nakano

Faculty of Agriculture, Niigata University, 2-8050 Ikarashi, Niigata 950-2181

Summary

An effective method for producing doubled haploid plants of the Asiatic hybrid lily 'Connecticut King' was established with an *in vitro* colchicine treatment of haploid calli. With an increase in the concentration and duration of colchicine treatment for the haploid calli, both the survival rate and the shoot regeneration from the calli decreased, but the frequency of diploid cells in the callus increased. Treatments of 0.25 and 0.5 mM colchicine for 48 or 72 hrs induced doubled haploid cells and shoot differentiation from calli. Haploid and diploid plantlets were regenerated from colchicine-treated calli, and only haploid plantlets were formed in colchicine-free treatments. This result suggests that these diploid plantlets originated from doubled haploid cells. Double haploids developed bulblets with more scaly leaves with longer stomatal guard cells than did the haploid plantlets.

Key Words: colchicine treatment, double haploid, haploid calli, *in vitro*, lily.

Introduction

Doubled haploid plant production is essential for the haploid breeding technique. We previously reported that haploid plantlets of the Asiatic hybrid lily 'Connecticut King' were obtained by anther culture (Han et al., 1997), but when these haploid plantlets were transplanted to soil, they grew poorly and produced no bulbs (unpublished data). This indicates that chromosome doubling of the haploids through chemical treatment should be carried out *in vitro* before transplanting to a greenhouse. *In vitro* colchicine treatment of haploid calli was effective in inducing chromosome doubling, through which doubled haploid maize and wheat plantlets were obtained successfully (Wan et al., 1989; Hassawi and Liang, 1991), respectively. In *Lilium* species, haploid (Sharp et al., 1971; Gu and Cheng, 1982; Han et al., 1997) and spontaneously doubled haploid plants (Arzate-Fernández et al., 1997) have been produced by anther culture in a few genotypes, but the production of doubled haploid plants derived from haploid calli by chemical treatment including colchicine has not been reported. In the present paper, we describe how we produced doubled haploid plantlets from colchicine-treated haploid calli of the Asiatic hybrid lily

'Connecticut King'.

Materials and Methods

1. Plant material and establishment of haploid callus line

Anthers of the Asiatic hybrid lily 'Connecticut King' ($2n = 2x = 24$) were cultured according to Han et al. (1997). A haploid callus line derived from a single anther, composed of more than 90% haploid cells, was selected and maintained in the dark at 25 °C on callus-proliferating MS medium (Murashige and Skoog, 1962). The medium consisting of 1 mg · liter⁻¹ picloram and 3% (w/v) sucrose was adjusted to pH 5.8 with 0.1 N NaOH before addition of 0.25% (w/v) gellan gum (Kanto Chemical Co., Inc., Japan). Forty ml of the MS medium was poured into a 100-ml Erlenmeyer flask and autoclaved for 15 min at 121 °C under a pressure of 1.2 kg · cm⁻². To proliferate the haploid callus line, the cultures were subcultured more than 6 times on the above callus-proliferation medium at intervals of 2-3 months. The ploidy level was examined on each subculture according to the method described below.

2. Colchicine treatment

An aqueous solution of 1 g · liter⁻¹ colchicine was prepared and added to a liquid callus-proliferation medium at varying concentration for each experiment. A 30-ml aliquot of the filter-sterilized medium was pipetted into a 100-ml Erlenmeyer flask. Haploid calli (about 3-5 mm diam.) were transferred into the liquid medium and incubated on a rotary shaker at 100 rpm in

Received; September 9, 1998. Accepted; February 13, 1999.

This work was supported by Grant-in-Aid for Science Research (No. 09460017) from Ministry of Education. Parts of this paper were presented at the 1997 autumn meeting of the Japanese Society for Horticultural Science.

* Corresponding author: e-mail: himesa@agr.niigata-u.ac.jp

the dark at 25 °C for 24, 48, and 72 hrs.

1) Effects of treatment duration

Calli were treated with 0.25 mM colchicine for 0 (control), 24, 48, and 72 hrs. They were then rinsed three times with a fresh liquid callus-proliferation medium without colchicine and transferred to the solid callus-proliferation medium. Three flasks, each containing 20 calli, were used for each treatment. These flasks were kept in the dark at 25 °C for 2 months. The survival rate of calli [(number of white or yellowish calli/ total number of calli in a flask) × 100] and ploidy level of cells in the calli were examined; 15 calli were transferred to a flask containing MS medium consisting of 0.1 mg · liter⁻¹ NAA, 0.01 mg · liter⁻¹ BA, 5% (w/v) sucrose, and 0.25% (w/v) gellan gum for plantlet regeneration. This medium is referred to the MS regeneration medium. Three flasks were used for each treatment. These flasks were kept at 25 °C under continuous illumination (50 μmol · m⁻² s⁻¹) with white fluorescent lamps (HITACHI FLR40SW). The number of regenerated shoots was determined after 2 months of culture.

2) Effects of concentration and treatment duration

Calli were treated with 0.25, 0.5, 1, and 2 mM colchicine for 0 (control), 24, 48, and 72 hrs. The calli, treated with colchicine, were rinsed three times with sterilized distilled water and transferred to the MS regeneration medium described above. Three flasks, each containing 10 calli per treatment, were used. The number of surviving calli, ploidy of calli, and percentage of regenerated plantlets were determined after 2 months of culture.

3. Cytological observations

The ploidy level of calli and regenerated plantlets were determined as follows: calli and root tips of plantlets were pretreated with 2 mM 8-hydroxyquinoline for 4 hrs, and then fixed in an acetic-alcohol solution (absolute alcohol : glacial acetic acid = 3 : 1, v/v) at room temperature for more than 24 hrs. The

samples were hydrolyzed with 1 N HCl at 60 °C for 10 to 15 min, rinsed with distilled water, stained with a 1% aceto-orcein solution using a squash method, and then observed under a light microscope.

4. Stomatal observations

The lengths of the stomatal guard cells were measured in 7 individual haploid and diploid plantlets. Three leaves were taken from each plantlet from which an abaxial, epidermal segments (about 1–2 mm²) were excised from the central portion of each leaf, and then placed on a microscope slide. The lengths of 10 stomatal guard cells per leaf, along the side facing the stomatal pore, were measured on the screen of a San-vision projection microscope.

Results

1. Effects of treatment duration of colchicine

There were no differences in their survival rate among calli treated at 0.25 mM colchicine for different durations but the ploidy of callus cells and the shoot regeneration rate differed (Table 1). The percentage of diploid callus-cell increased but the percentage of the shoot regeneration from the calli decreased as the treatment duration was lengthened from 24 to 72 hrs.

When shoots, regenerated from colchicine-treated calli, were transferred to a fresh MS regeneration medium, plantlets composed of scales and scaly leaves developed in about 2 months. When the chromosome numbers of root tip cells in 10 plantlets were examined, 4 plantlets were haploids (2n = 12, Fig. 1A) and 6 plantlets were diploids (2n = 24, Fig. 1B). No chimeric or tetraploid plants were found.

2. Effects of concentration and duration of colchicine treatment

The survival rate of calli ranged from 56.7 to 96.7% in each treatment; the percentage of diploid cells in callus increased from 42.8 to 79.0% when haploid calli were treated with 0.25 to 2 mM colchicine for 48 to 72 hrs (Table 2). But the callus survival and shoot regeneration

Table 1. Effect of treatment duration of 0.25 mM colchicine on the survival rate, ploidy level of callus cells, and shoot regeneration of callus in the Asiatic hybrid lily 'Connecticut King'.^z

Duration of treatment (hr)	Survival rate of callus ^y (%)	% of callus-cell ploidy ^x		% of shoot regeneration on callus ^w
		Haploid	Diploid	
0 (control)	100a	92.3a	7.7a	42.2ab
24	100a	75.5ab	24.5ab	62.2a
48	100a	57.7bc	42.3bc	56.6a
72	100a	37.7c	62.3c	34.3b

^z Averages followed by the different letters in each column are significantly different at 0.05 level of probability as determined by Duncan's multiple range test.

^y Each value is the average of three flasks, and each flask had 20 calli.

^x Each value is the average of five calli, and over 40 cells at metaphase were observed in each callus.

^w Each value is the average of three flasks, and each flask had 15 calli.

Table 2. Effects of combinations of colchicine concentration and treatment duration on the survival rate, ploidy level of callus cells, and shoot regeneration of callus in the Asiatic hybrid lily 'Connecticut King'.^z

Combinations of colchicine treatment		Survival rate of callus ^y (%)	% of callus - cell ploidy ^x		% of shoot regeneration on callus ^y
Concentration (mM)	Duration (hr)		Haploid	Diploid	
0 (control)	0	96.7a	88.2a	11.8a	46.7a
0.25	24	96.7a	77.6ab	22.4ab	36.7ab
0.25	48	80.0abc	57.2bc	42.8bc	26.7abc
0.25	72	63.0cde	48.2bcd	51.8bcd	16.7cde
0.5	24	86.7ab	64.4abc	35.6abc	36.7ab
0.5	48	80.0abc	55.0bcd	45.0bcd	20.0bcde
0.5	72	60.0de	39.6cd	60.4cd	13.3de
1.0	24	80.0abc	58.0abc	42.0abc	33.3abc
1.0	48	76.7bcd	49.4bcd	50.6bcd	16.7cde
1.0	72	60.0de	34.4cd	65.6cd	13.3de
2.0	24	76.7bcd	53.2bcd	46.8bcd	16.7cde
2.0	48	70.0bcde	44.0cd	56.0cd	10.0de
2.0	72	56.7e	21.0d	79.0d	6.7e

^z Averages followed by the different letters in each column are significantly different at 0.05 level of probability as determined by Duncan's multiple range test.

^y Each value is the average of three flasks, and each flask had 10 calli.

^x Each value is the average from five calli, and over 40 cells at metaphase were observed in each callus.

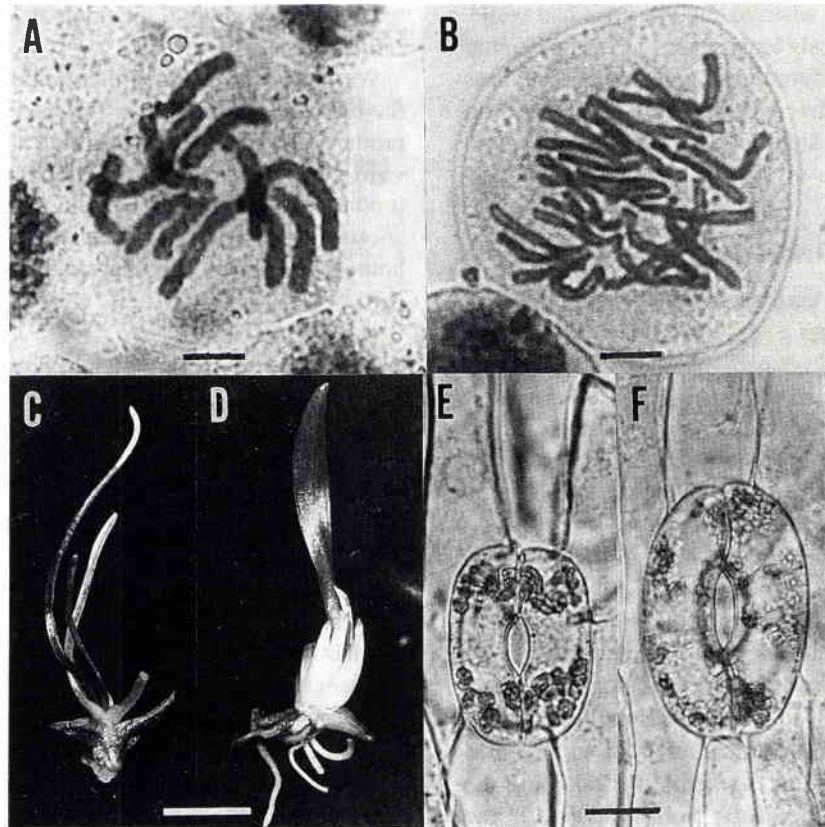


Fig. 1. Photomicrographs of (A) haploid chromosomes ($2n = 12$, bar = $10 \mu\text{m}$) and (B) diploid chromosomes ($2n = 24$, bar = $15 \mu\text{m}$), photographs of (C) haploid and (D) diploid plantlets regenerated from colchicine-treated haploid calli (bar = 1 cm), and photomicrographs of (E) haploid and (F) diploid stomatal guard cells (bar = $20 \mu\text{m}$).

Table 3. Comparison of stomatal guard cell length between haploid and diploid plantlets in the Asiatic hybrid lily 'Connecticut King'.

Ploidy level of plantlets	Total number of plantlets observed	Stomatal guard cell length (μm) ^z	
		Range of variation	Average ^y
Haploid	7	46.4–52.0	49.2a
Diploid	7	64.7–70.8	68.4b

^z Measurements of stomatal guard cell length were made on 30 stomatal guard cells from three leaves in each plantlet.

^y Averages followed by the different letters are significantly different at 0.01 level of probability as determined by t-test.

rates decreased when the duration of colchicine treatment was lengthened from 24 to 72 hrs. At 2 mM colchicine, only 6.7 to 16.7% of the calli regenerated shoots.

When the ploidy levels of 16 regenerated plantlets in Table 2 were examined, 14 plantlets were diploids, and 2 plantlets were haploids. No diploid plantlets were found in the control.

3. Observation of morphology and stomata

There were a few differences in the morphological characteristics of the haploid and diploid plantlets. Haploid plantlets had poorly grown scales with long scaly leaves (Fig. 1C), whereas diploid ones had well-developed scales and scaly leaves (Fig. 1D). The haploid and diploid plantlets differed in stomatal guard cell sizes (Figs 1E and 1F). The average stomatal length on individual plants was significantly smaller in haploid (49.2 μm) than it was in diploid (68.4 μm) plantlets (Table 3).

Discussion

Colchicine has been used as an effective chemical in obtaining a high frequency of doubled haploid plantlets in anther-derived haploid calli in maize (Wan et al., 1989) and wheat (Hassawi and Liang, 1991). This report is the first to show that doubled haploid plantlets can be effectively produced by an *in vitro* colchicine treatment on haploid calli derived from anther cultures of lily.

But colchicine also has a negative toxic effect, decreasing the survival rate and regeneration rate of cultures (Jähne and Lörz, 1995; Cohen and Yao, 1996; Van Duren et al., 1996; Song et al., 1997). We found that both callus survival and shoot formation decreased as the concentration and duration of colchicine treatment increased (Table 1 and Table 2). Based on these results, we concluded that the treatment of 0.25 to 0.5 mM colchicine for 48 to 72 hrs is suitable for doubling the haploid cells and inducing shoots in haploid calli of the Asiatic hybrid lily 'Connecticut King'.

When leaves (Espino and Vaequez, 1981) and plantlets (Hsu et al., 1991) are cultured *in vitro* with colchicine, chimeric plants are often regenerated. Our study showed that colchicine-treated haploid calli regenerated

haploid and diploid plantlets, but not chimeric ones, indicating that the doubled haploid plants of 'Connecticut King' can be produced from calli while avoiding the regeneration of chimeric plants.

Alternative indicators of the ploidy level, such as stomatal guard cell length (Tan and Dunn, 1973; Santen and Casler, 1986; Borrino and Powell, 1988; Mishra, 1997), have been used, because chromosome counting is laborious when large numbers of plants must be examined. There are significant differences in stomatal guard cell lengths of diploid and tetraploid in *Lilium* species (Iizuka and Ikeda, 1968), which our study confirmed (Table 3). Hence, this method could be used to distinguish diploid plantlets from haploid ones in *Lilium* species.

We have demonstrated that doubled haploid plants of the Asiatic hybrid lily 'Connecticut King' can be produced by treating the haploid calli with colchicine *in vitro*. This method may be applicable to the *in vitro* production of doubled haploid plants in other *Lilium* species and will enable the production of a completely homozygous line of lily in a single generation.

Acknowledgements

The authors wish to thank Prof. S. M. Goldstein, Keiwa-gakuen University for critically reading the manuscript and correcting the English text.

Literature Cited

- Arzate-Fernández, A.-M., T. Nakazaki, H. Yamagata and T. Tanisaka. 1997. Production of doubled-haploid plants from *Lilium longiflorum* Thunb. anther culture. *Plant Sci.* 123:179–187.
- Borrino, E. M. and W. Powell. 1988. Stomatal guard cell length as an indicator of ploidy in microspore-derived plants of barley. *Genome* 30: 158–160.
- Cohen, D. and J. L. Yao. 1996. *In vitro* chromosome doubling of nine *Zantedeschia* cultivars. *Plant Cell Tiss. Org. Cult.* 47: 43–49.
- Espino, E. J. and A. M. Vaequez. 1981. Chromosome numbers of *Saintpaulia ionantha* plantlets regenerated from leaves cultured *in vitro* with caffeine and colchicine. *Euphytica* 30: 847–853.
- Gu, Z. P. and K. C. Cheng. 1982. Studies on induction of

- pollen plantlets from the anther cultures of lily. *Acta Bot. Sin.* 24: 28-32.
- Han, D. S., Y. Niimi and M. Nakano. 1997. Regeneration of haploid plants from anther cultures of the Asiatic hybrid lily 'Connecticut King'. *Plant Cell Tiss. Org. Cult.* 47: 153-158.
- Hassawi, D. S. and G. H. Liang. 1991. Antimitotic agents: effects on double haploid production in wheat. *Crop Sci.* 31: 723-726.
- Hsu, J. Y., P. C. Lai, C. C. Yeh and H. S. Tsay. 1991. Breeding for all-male plants through anther culture in asparagus. II. Chromosome doubling techniques of anther-derived haploid plant. *Jour. Agri. Res. China* 40: 102-114.
- Iizuka, M. and A. Ikeda. 1968. Induction of polyploidy in *Lilium formosanum* Wallace. *Japan. J. Genetics* 43: 95-101.
- Jähne, A. and H. Lörz. 1995. Cereal microspore culture. *Plant Sci.* 109: 1-12.
- Mishra, M. K. 1997. Stomatal characteristics at different ploidy levels in *Coffea* L. *Ann. Bot.* 80: 689-692.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-479.
- Santen E. V. and E. V. Casler. 1986. Evaluation of indirect ploidy indicators in *Dactylis* L. subspecies. *Crop. Sci.* 26: 848-852.
- Sharp, W. R., R. S. Raskin and H. E. Sommer. 1971. Haploidy in *Lilium*. *Phytomorphol.* 21: 334-335.
- Song, P., W. Kang and E. B. Peffley. 1997. Chromosome doubling of *Allium fistulosum* × *A. cepa* interspecific F₁ hybrids through colchicine treatment of regenerating callus. *Euphytica* 93: 257-262.
- Tan G. Y. and G. M. Dunn. 1973. Relationship of stomatal length and frequency and pollen grain diameter to ploidy level in *Bromus inermis* Leyss. *Crop. Sci.* 13: 332-334.
- van Duren, M., R. Morpurgo, J. Dolezel and R. Afza. 1996. Induction and verification of autoteraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. *Euphytica* 88: 25-34.
- Wan, Y., J. F. Petolino and J. M. Widholm. 1989. Efficient production of doubled haploid plants through colchicine treatment of anther-derived maize callus. *Theor. Appl. Genet.* 77: 889-892.

薬由来の半数性カルスのコルヒチン処理によるアジアティックハイブリッドユリ 'コネチカットキング' の倍加半数体の作出

韓 東生・新美芳二・中野 優

新潟大学農学部 950-2181 新潟市五十嵐2-8050

摘 要

本研究ではコルヒチン処理によるアジアティックハイブリッドユリ 'コネチカットキング' の倍加半数体植物の獲得方法について検討した。薬由来の半数性カルスの生存率とシュート再生率は、コルヒチン濃度の上昇および処理時間の延長にともなって低下したが、2倍性カルス細胞の割合は増加した。半数性カルス細胞の倍加とシュート再生率には、0.25mMと0.5mMのコルヒチンを用いた48時間または72時間処理が良

好であることが明らかとなった。コルヒチン処理された半数性カルスは半数体と2倍体植物を再生したが、対照区(コルヒチン無処理)のカルスは半数性植物のみを再生したことから、これらの2倍体植物は半数性細胞が倍加されたことにより得られた倍加半数体植物であることが推察された。倍加半数体植物では、葉の気孔サイズは半数体植物のものより大きく、子球もよく発達した。