Chromosome doubling of *Tricyrtis formosana* by *in vitro* spindle toxin treatments

Masaru NAKANO[1]*, Chiaki GOTO[1], Miki YAMAKAWA[1], Mitsuyo ISHIBE[1], Toshiya INAMURA[2] and Masahiro OTANI[1]

(Received November 16, 2017)

Summary

Tricyrtis formosana, a liliaceous perennial plant, is native to Taiwan and the Ryukyu Islands. This species is most popular among *Tricyrtis* spp. as an ornamental plant for pot and garden uses. In the present study, chromosome doubling of *T. formosana* ($2n=2x=26$) was examined for widening its variability in horticultural traits by *in vitro* spindle toxin treatments. Nodal segments harvested from *in vitro*-grown plantlets were treated for 24 h with 100, 500 or 1000 mg L^{-1} colchicine (COL), 10, 20 or 50 mg L^{-1} oryzalin (ORY), or 1, 5, 10 or 50 mg L^{-1} amiprophos-methyl (APM). In 100 mg L^{-1} COL, 10 mg L^{-1} ORY and 1 mg L^{-1} APM treatments, over 85% of nodal segments survived and over 20 elongating shoots were obtained from 30 nodal segments two months after treatment. For all three spindle toxins, the percentage of surviving segments and the number elongating shoots decreased as the treatment concentration increased. Flow cytometry (FCM) analysis of plantlets regenerated from spindle toxin-treated nodal segments showed that the highest percentage of chromosome-doubled plantlets (71.4%) was obtained by 10 mg L^{-1} APM treatment. However, most plantlets with chromosome doubling were ploidy chimera ($2x+4x$), and only a few tetraploids ($4x$) were obtained. Further studies are necessary to establish an efficient chromosome doubling system without regeneration of ploidy chimeras.

Bull.Facul.Agric.Niigata Univ., 70:9-13, 2018

Key words : amiprophos-methyl, flow cytometry analysis, liliaceous ornamental plant, ploidy chimera, tetraploid

Tricyrtis formosana, a liliaceous perennial plant, is native to Taiwan and the Ryukyu Islands. This species is most popular among *Tricyrtis* spp. as an ornamental plant for pot and garden uses because of its vigorous growth and beautiful flowers with various colors. A number of *T. formosana* cultivars have so far been bred and distributed in Japan (Nagamura, 1989). However, for more popularization of *T. formosana*, it is desired to produce novel cultivars with attractive traits such as larger flowers and compact growth habit.

Polyploidization often causes horticulturally attractive traits such as larger flowers, deeper green leaves, thicker stems and compact growth habit (Lindsay *et al*., 1994; Gao *et al*., 1996; Takamura and Miyajima, 1996; Tang *et al*., 2010; Nonaka *et al*., 2011; Nakano *et al*. 2012; Yamakawa *et al*., 2015). In *Tricyrtis*., tetraploid plants of cv. Shinonome were spontaneously regenerated as chromosome-doubled somaclonal variants from one-year-old embryogenic callus cultures (Nakano *et al*., 2006). They showed longer shoots, thicker stems and larger flowers compared with the diploid control. However, regeneration frequency of tetraploid somaclonal variants was relatively low. In addition, undesirable severely dwarf somaclonal variants with crimped leaves and malformed flowers were also regenerated from the same embryogenic callus cultures (Nakano *et al*., 2006).

In the present study, we examined artificial chromosome doubling of *T. formosana* for widening their variability in horticultural traits by *in vitro* spindle toxin treatments of nodal segments. Effect of three different spindle toxins, APM, COL and ORY, was investigated on the survival of nodal segments and chromosome doubling of nodal segment-derived plantlets.

MATERIALS AND METHODS

Plant material and plantlet culture

T. formosana cv. Seiryu ($2n=2x=26$) was used in the present study. Potted plants were cultivated in the greenhouse without heating (Nakano *et al*., 2004).

For establishing *in vitro* plantlet cultures, vigorously growing stems were harvested from potted plants in late spring. After removing leaves, stems were surface-sterilized in a sodium hypochlorite solution containing 1% active chlorine for 10 min, followed by 3 rinses with sterile, distilled water. Nodal segments (ca. 10 mm in length) were placed on a plantlet culture medium [half-strength Murashige and Skoog (1962) (MS) medium supplemented with 30 g L^{-1} sucrose and 2 g L^{-1} gellan gum, pH 5.7]. Axillary bud-derived plantlets were subcultured every 2 months by transferring nodal or apical segments (ca. 10 mm in length) to fresh medium of the same composition. All cultures before and after spindle toxin treatment were maintained at 25℃ under continuous illumination with fluorescent light (50 μmol m^{-2} s^{-1}).

[1] Graduate School of Science and Technology, Niigata University

[2] Faculty of Agriculture, Niigata University

* Corresponding author: mnakano@agr.niigata-u.ac.jp

Spindle toxin treatment and plantlet regeneration

Three spindle toxins, COL (Kanto Chemical Co., Inc., Japan), ORY (AccuStandard Inc., USA) and APM (Hayashi Pure Chemical Ind., Ltd., Japan), were used in the present study. Stock solutions of these spindle toxins were prepared in water-free DMSO. Liquid plantlet culture media supplemented with 100, 500 or 1000 mg L^{-1} COL, 10, 20 or 50 mg L^{-1} ORY, or 1, 5, 10 or 50 mg L^{-1} APM were used as spindle toxin treatment solutions. Liquid plantlet culture medium without spindle toxins was used as a control. Nodal segments (ca. 10 mm in length) were harvested from *in vitro*-grown plantlets 1-2 months after subculture. After removing leaves, nodal segments were soaked in spindle toxin treatment solutions and incubated on a rotary shaker (120 cycles min^{-1}) at 25℃ for 24 h. Nodal segments were then rinsed three times with sterile, distilled water and cultured on the plantlet culture medium. Two months after spindle toxin treatment, the number of surviving nodal segments and the number of axillary bud-derived elongating shoots (over 10 mm in length) were recorded.

Within 3 months after spindle toxin treatment, shoots elongating over 20 mm in length were isolated from the nodal segments and transferred to fresh medium of the same composition.

FCM analysis and chromosome observation

Ploidy level of spindle toxin treatment-derived plantlets was estimated by FCM analysis of leaf tissues as previously described (Saito *et al*., 2003). At least 2000 nuclei were examined for each plantlet. Chromosome observation in root-tip cells of some plantlets was performed as previously described (Nakano *et al*., 2006).

RESULTS AND DISCUSSION

Table 1 shows effect of various spindle toxin treatments on survival and development of nodal segments two months after treatment. In the control treatment without spindle toxins, almost all of nodal segments survived, and totally 26 elongating shoots were obtained from 30 nodal segments. After spindle toxin treatment, some nodal segments turned brown and died, but some segments survived and developed shoots. In 100 mg L^{-1} COL, 10 mg L^{-1} ORY and 1 mg L^{-1} APM treatments, over 85% of nodal segments survived and over 20 elongating shoots were obtained from 30 nodal segments. For all three spindle toxins, the percentage of surviving segments and the number elongating shoots decreased as the spindle toxin concentration increased. All nodal segments treated with 50 mg L^{-1} APM died within one month after treatment.

Irrespective of the kind and concentration of spindle toxin, all of elongating shoots produced adventitious roots and developed into plantlets within one month after isolation from the nodal segments and transfer to fresh medium. In order to estimate the ploidy level of these plantlets, FCM analysis of leaf tissues was carried out (Fig. 1). In the diploid mother plant (2*x*), histogram showed a single peak corresponding to nuclei in the G0/G1 phase of the cell cycle. Neither ploidy chimera nor polysomaty were found as in the case of *Tricyrtis* cv. Shinonome (Nakano *et al*., 2006). In the control treatment without spindle toxins, the G0/G1 peak of all plantlets appeared at almost the same position as the mother plant, indicating that they were diploid (2*x*). On the other hand, a single G0/G1 peak corresponding to tetraploid (4*x*) appeared in histograms of one plantlet derived from 5 mg L^{-1} APM treatment and two plantlets derived from 10 mg L^{-1} APM treatment. These results were confirmed by chromosome observation in root-tip cells.

Table 1. Effect of *in vitro* spindle toxin treatments of nodal segments on survival of nodal segments and chromosome doubling of nodal segment-derived plantlets in *Tricyrtis formosana* cv. Seiryu.

Spindle toxins	Concentration (mg L^{-1})	No. of nodal segments treated	% of surviving nodal segments [a]	Total no. of elongating shoots [a]	No. of plantlets showing each ploidy level [b]			% of tetraploid + ploidy chimera plantlets [b]
					Diploid (2*x*)	Tetraploid (4*x*)	Ploidy chimera (2*x*+4*x*)	
Control	-	30	93.3	26	26	0	0	0
COL	100	30	90.0	23	22	0	1	4.3
	500	30	70.0	19	16	0	3	15.8
	1000	30	66.7	12	9	0	3	25.0
ORY	10	30	86.7	23	23	0	0	0
	20	30	46.7	14	12	0	2	14.3
	50	30	26.7	8	7	0	1	12.5
APM	1	30	86.7	25	21	0	4	16.0
	5	30	70.0	13	8	1	4	38.5
	10	30	33.3	7	2	2	3	71.4
	50	30	0	0	0	0	0	0

[a] Percentage of surviving nodal segments and total number of elongating shoots were recorded two months after spindle toxin treatment.
[b] Ploidy level was estimated by FCM analysis four months after spindle toxin treatment.

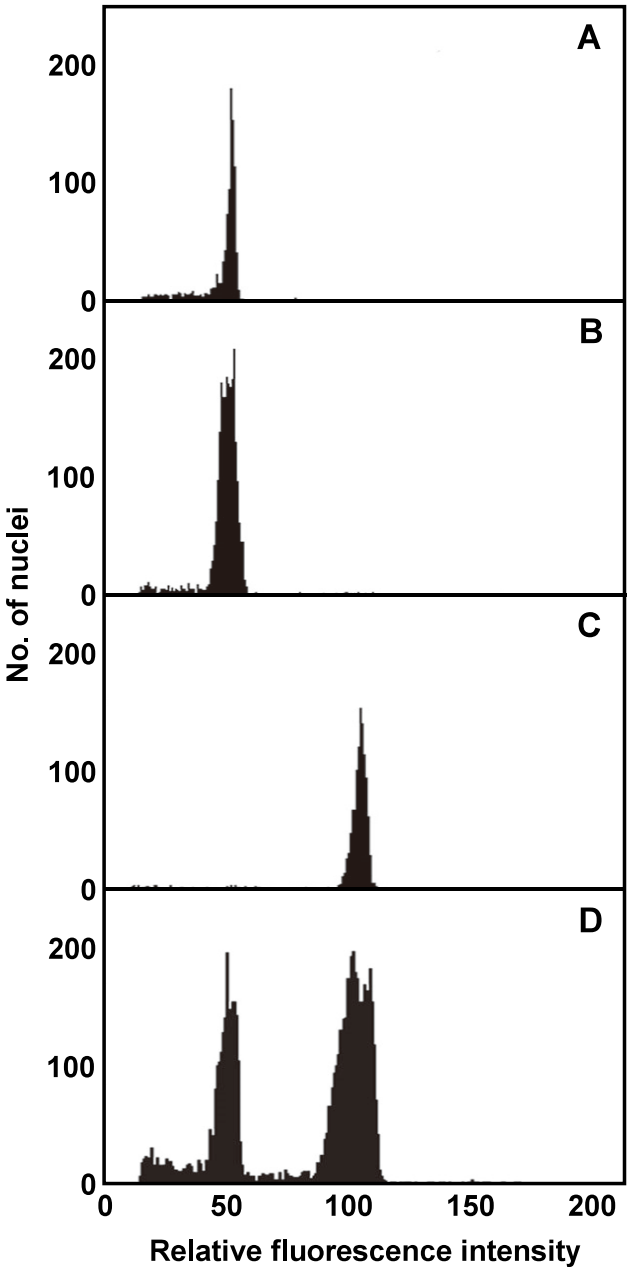

Fig 1. Histograms from FCM analysis of the nuclear DNA content in *Tricyrtis formosana* cv. Seiryu. **A**, Diploid mother plant (2*x*); **B**, diploid plantlet (2*x*) derived from the control treatment without spindle toxins; **C**, tetraploid plantlet (4*x*) derived from 10 mg L^{-1} ORY treatment; **D**, ploidy chimera plantlet (2*x*+4*x*) derived from 10 mg L^{-1} ORY treatment.

Plantlets estimated to be diploid and tetraploid had 2*n*=26 and 2*n*=52 chromosomes, respectively (data not shown).

In some plantlets derived from 100, 500 and 1000 mg L^{-1} COL treatments, 20 and 50 mg L^{-1} ORY treatments, and 1, 5 and 10 mg L^{-1} APM treatments, FCM histograms showed two G0/G1 peaks corresponding to diploid and tetraploid, indicating that they were ploidy chimera (2*x*+4*x*). Ploidy level of these ploidy chimeras was unstable, and they tended to become solid polyploids when successively subcultured by transferring apical or nodal segments to fresh medium. For example, ploidy chimeras with relatively high proportions of diploid cells turned solid diploids after two or three subcultures. Similar observations have been reported for *Lychnis* spp. (Nonaka *et al*., 2011).

In *T. formosana* cv. Seiryu, chromosome doubling could be induced by all three spindle toxins, among which APM seemed to be more effective than the other two spindle toxins (Table 1). The highest percentage of chromosome-doubled (tetraploid + ploidy chimera) plantlets (71.4%) was obtained by 10 mg L^{-1} APM treatment. In addition, solid tetraploid plantlets could be obtained only by APM treatments. It has been reported that the kind of spindle toxin often affect the efficiency of chromosome doubling (Sree Ramulu *et al*., 1991; Yahata *et al*., 2004; Nonaka *et al*., 2011; Rodrigues *et al*., 2011; Yamakawa *et al*., 2016). Although COL has been used most commonly (Hancock, 1997), APM has been reported to be more efficient than COL for chromosome doubling in several plant species (Sree Ramulu *et al*., 1991; Yahata *et al*., 2004; Rodrigues *et al*., 2011).

In the present study, chromosome doubling of *T. formosana* cv. Seiryu was successfully induced by *in vitro* spindle toxin treatments of nodal segments, and totally 24 chromosome-doubled (tetraploid + ploidy chimera) plantlets were obtained. However, most of them were unstable ploidy chimeras, and only three were desirable solid tetraploids. Therefore, further studies are necessary to establish an efficient chromosome doubling system without regeneration of ploidy chimeras.

REFERENCE

Gao, S. L., D. N. Zhu, Z. H. Cai and D. R. Xu. 1996. Autotetraploid plants from colchicine-treated bud culture of *Salvia miltiorrhiza* Bge. *Plant Cell Tiss. Org. Cult*., **47**: 73-77.

Hancock, J. F. 1997. The colchicine story. *HortScience*, **32**: 1011-1012.

Lindsay, G. C., M. E. Hopping and I. E. W. O'Brien. 1994. Detection of protoplast-derived DNA tetraploid Lisianthus (*Eustoma grandiflorum*) plants by leaf and flower characteristics and by flow cytometry. *Plant Cell Tiss. Org. Cult*., **38**: 53-55.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*., **15**: 473-497.

Nagamura, Y. 1989. *Tricyrtis* Wall., nom. cons. In: Tsukamoto, Y., ed. The grand dictionary of horticulture, vol. 4. pp. 431-433, Shogakukan, Tokyo (in Japanese).

Nakano, M., K. Mizunashi, S. Tanaka, T. Godo, M. Nakata and H. Saito. 2004. Somatic embryogenesis and plant regeneration from callus cultures of several species in the genus *Tricyrtis*. *In Vitro Cell. Dev. Biol. Plant*, **40**: 274-278.

Nakano, M., T. Nomizu, K. Mizunashi, M. Suzuki, S. Mori, S. Kuwayama, M. Hayashi, H. Umehara, E. Oka, H. Kobayashi, M. Asano, S. Sugawara, H. Takagi, H. Saito, M. Nakata, T. Godo, Y. Hara and J. Amano. 2006. Somaclonal variation in *Tricyrtis hirta* plants regenerated from 1-year-old embryogenic callus cultures. *Sci. Hortic*., **110**: 366-371.

Nakano, M., H. Tasaki, T. Nonaka, D.-S. Han and T. Godo. 2012. Chromosome doubling of Japanese native *Lychnis* spp. by *in vitro* oryzalin treatment. *Bull. Fac. Agric. Niigata Univ*., **64**: 101-105.

Nonaka, T., E. Oka, M. Asano, S. Kuwayama, H. Tasaki, D.-S. Han, T. Godo and M. Nakano. 2011. Chromosome doubling of *Lychnis* spp. by *in vitro* spindle toxin treatment of nodal segments. *Sci. Hortic*., **129**: 832-839.

Rodrigues, F. A., J. D. R. Soares, R. R. dos Santos, M. Pasqual1 and S. O. Silva. 2011. Colchicine and amiprophos-methyl (APM) in polyploidy induction in banana plant. *Afr. J. Biotechnol*., **10**: 13476-13481.

Saito, H., K. Mizunashi, S. Tanaka, Y. Adachi and M. Nakano. 2003. Ploidy estimation in *Hemerocallis* species and cultivars by flow cytometry. *Sci. Hortic*., **97**: 185-192.

Sree Ramulu, K., H. A. Verhoeven and P. Dijkhuis. 1991. Mitotic blocking, micronucleation, and chromosome doubling by oryzalin, amiprophos-methyl, and colchicine in potato. *Protoplasma*, **160**: 65-71.

Takamura, T. and I. Miyajima, 1996. Colchicine induced tetraploids in yellow-flowered cyclamens and their characteristics. *Sci. Hortic*., **65**: 305-312.

Tang, Z.-Q., D.-L. Chen, Z.-J. Song and Y.-C. He. 2010. In vitro induction and identification of tetraploid plants of *Paulownia tomentosa*. *Plant Cell Tiss. Org. Cult*., **102**: 213-220.

Yahata, S., S. Sato, H. Ohara and H. Matsui. 2004. Induction of tetraploid in loquat with amiprophos-methyl and colchicine. *Hort. Res*., **3**: 339-344 (in Japanese with English summary).

Yamakawa, M., T. Kishimoto, T. Sato, T. Saito, J. Amano, M. Otani and M. Nakano. 2016. Chromosome doubling of a colchicaceous intergeneric hybrid by spindle toxin treatments. *Bull. Fac. Agric. Niigata Univ*., **68**: 25-30.

タイワンホトトギスにおける培養物の紡錘糸形成阻害剤処理による染色体倍加

中野　優[1*]・後藤千晶[1]・山川美樹[1]・石部万葉[1]・稲村俊哉[2]・大谷真広[1]

（平成29年11月16日受付）

要　約

タイワンホトトギス（*Tricyrtis formosana*；2*n*=2*x*=26）はユリ科に属する多年草で、台湾や日本の南西諸島に自生している。ホトトギス属植物の中では、鉢物・庭植え用花き園芸植物として最も人気がある。本研究では、タイワンホトトギスの園芸形質の拡大を目的として、培養物の紡錘糸形成阻害剤処理による染色体倍加を試みた。培養小植物体から節切片を調製し、様々な濃度のコルヒチン（COL；100、500または1000 mg L^{-1}）、オリザリン（ORY；10、20または50 mg L^{-1}）、またはアミプロホスメチル（APM；1、5、10または50 mg L^{-1}）で24時間処理した。100 mg L^{-1} COL 処理区、10 mg L^{-1} ORY 処理区および 1 mg L^{-1} APM 処理区においては、処理２ヵ月後に85% 以上の節切片が生存し、また、30節切片から20本以上の伸長シュートが得られた。いずれの紡錘糸形成阻害剤においても、節切片生存率および伸長シュート数は処理濃度の上昇とともに減少した。紡錘糸形成阻害剤処理後の節切片から再生した小植物体についてフローサイトメトリー分析を行ったところ、染色体倍加を示す小植物体の割合は10 mg L^{-1} ORY 処理区で最も高かった（71.4%）。しかしながら、染色体倍加を示す小植物体のほとんどは倍数性キメラ（2*x*+4*x*）であり、四倍体小植物体（4*x*）はわずかしか得られなかった。今後は、倍数性キメラ個体の再生を抑制できる染色体倍加方法の検討が必要である。

新大農研報, 70:9-13, 2018

キーワード：アミプロホスメチル、倍数性キメラ、フローサイトメトリー分析、ユリ科花き園芸植物、四倍体

[1] 新潟大学大学院自然科学研究科

[2] 新潟大学農学部

* 代表著者 : mnakano@agr.niigata-u.ac.jp