Chromosome doubling of a colchicaceous intergeneric hybrid by spindle toxin treatments

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Summary

The family Colchicaceae contains some important ornamental plants, such as *Gloriosa* spp. and *Sandersonia aurantiaca*. We have produced various intergeneric hybrid plants in this family in order to obtain wide variability in horticultural traits and to develop novel cultivars. In the present study, we examined chromosome doubling of a diploid intergeneric hybrid between *L. modesta* and *G. superba* 'Lutea' (Lit × Gsu-1; $2n=2x=22$) for fertility restoration and for further widening the variability. Shoot apical segments harvested from *in vitro* shoot cultures were treated with various concentrations of amiprophos-methyl (APM; 10, 20 or 40 mg L^{-1}), colchicine (COL; 100, 500 or 1000 mg L^{-1}) or oryzalin (ORY; 5, 10 or 20 mg L^{-1}) for 24 h. Two months after spindle toxin treatment, the highest percentage apical segments with shoot elongation (80.0%) was obtained in 10 mg L^{-1} APM treatment. Flow cytometry analysis of elongated shoots showed that a solid tetraploid ($4x$) was obtained from 40 mg L^{-1} APM treatment and ploidy chimeras ($2x+4x$) were obtained from treatments with 10 or 20 mg L^{-1} APM and 5 or 10 mg L^{-1} ORY. Tetraploid and polyploidy chimera shoots showed compact forms with reduced internodes compared with diploid shoots.

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Key words : amiprophos-methyl, flow-cytometry analysis, *Gloriosa superba, Littonia modesta,* oryzalin

Gloriosa spp., *Littonia modesta* and *Sandersonia aurantiaca*, all belonging to the family Colchicaceae, are tuberous plants native to South Africa. *Gloriosa* spp. and *S. aurantiaca* are cultivated worldwide as ornamental plants because of their beautiful and unique flowers (Eason *et al*., 1995; Nakamura *et al*., 2005). In order to obtain wide variability in horticultural traits and to develop novel cultivars, we have produced various intergeneric hybrids among these plants via ovule culture (Kuwayama *et al*., 2005; Nakamura *et al*., 2005; Amano *et al*., 2007, 2008, 2009). Although some hybrid plants were horticulturally attractive, all hybrid plants showed very low or no pollen fertility (0−2.4%) as assessed with acetocarmine staining (Amano *et al*., 2008, 2009). Thus, these hybrid plants are unusable as materials for further cross-breeding.

Chromosome doubling by spindle toxin treatment has been carried out for fertility restoration of various wide hybrids, such as interspecific hybrids in *Vaccinium* (Miyashita *et al*., 2009) and *Dianthus* (Nimura, 2006), and intergeneric hybrids between *Lolium* and *Festuca* (Zwierzykowski, 1980). Chromosome doubling has also been used as a breeding tool for gaining novel characteristics in ornamental plants (Väinölä, 2010). Polyploidization often causes horticulturally attractive traits such as compact growth habit, thicker stems, deeper green leaves and larger flowers (Gao *et al*., 1996; Takamura and Miyajima, 1996; Nakano *et al*., 2006; Nonaka *et al*., 2011).

Several spindle toxins, such as amiprophos-methyl (APM), colchicine (COL) and oryzalin (ORY), have so far been used for chromosome doubling in plants. Although COL has been used most commonly (Hancock, 1997), recent studies have reported that ORY and APM showed higher effects on chromosome doubling with less damage to plant tissues compared with COL in various plant species (Ramulu *et al*., 1991; Tosca *et al*., 1995; Thao *et al*., 2003; Sakhanokho *et al*., 2009; Nonaka *et al*., 2011). In colchicaceous plants, tetraploid *S. aurantiaca* has successfully been produced by *in vitro* ORY treatment of shoots (Burge *et al*., 2008).

In the present study, we examined the development of an efficient chromosome doubling system in colchicaceous intergeneric hybrids by *in vitro* spindle toxin treatments of shoot apical segments. Effect of three different spindle toxins, APM, COL and ORY, was investigated on elongation of apical segment-derived shoots and chromosome doubling of apical segment-derived shoots by using a diploid intergeneric hybrid between *L. modesta* and *G. superba* 'Lutea'.

MATERIALS AND METHODS

Plant material and shoot culture

A diploid intergeneric hybrid of *L. modesta* × *G. superba* 'Lutea' (Lit × Gsu-1; $2n=2x=22$) produced via ovule culture

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(Kuwayama *et al*., 2005; Amano *et al*., 2007, 2008, 2009) was used in present study. Tubers were planted in pots containing vermiculite and cultivated at room temperature (20－25℃).

Shoots tips with an apical meristem (ca. 5 cm in length) were isolated from tuber-derived shoots and surface-sterilized in a sodium hypochlorite solution containing 1% active chlorine for 5 min followed by three rinses with sterile, distilled water. Shoot tips were placed on Medium I consisting of MS basal salts and vitamins (Murashige and Skoog, 1962), 1 mg L^{-1} α-naphthaleneacetic acid (NAA), 0.2 mg L^{-1} benzyladenine (BA), 30 g L^{-1} sucrose, and 2 g L^{-1} gellan gum, pH 5.8. After one month, elongated shoots were transferred to Medium II consisting of MS basal salts and vitamins, 0.5 mg L^{-1} NAA, 2.5 mg L^{-1} BA, 30 g L^{-1} sucrose, and 2 g L^{-1} gellan gum, pH 5.8. One month later, shoots were further transferred to Medium III consisting of MS basal salts and vitamins, 0.1 mg L^{-1} NAA, 10 mg L^{-1} BA, 30 g L^{-1} sucrose, and 2 g L^{-1} gellan gum, pH 5.8. Subculture was performed every two months by transferring shoots to fresh Medium III. All cultures in the present study were maintained at 25℃ under continuous illumination with fluorescence light (35 μmol m^{-2} s^{-1}).

Spindle toxin treatments

Three spindle toxins, APM (Duchefa Biochemi, The Netherlands), COL (Wako Pure Chemical, Japan) and ORY (Wako Pure Chemical, Japan), were used in the present study. Stock solutions of these spindle toxins were prepared in water-free dimethyl sulfoxide (DMSO). Liquid Medium IIIs supplemented with 10, 20 or 40 mg L^{-1} APM, 100, 500 or 1000 mg L^{-1} COL, or 5, 10 or 20 mg L^{-1} ORY were used as spindle toxin treatment solutions. Liquid Medium III without any spindle toxins was used as a control.

Shoot apical segments (ca. 2 cm in length) were harvested from shoot cultures two months after subculture. They were soaked in spindle toxin treatment solutions and incubated on a rotary shaker (120 rpm) at 25℃ for 24 h. Apical segments were then rinsed three times with sterile, distilled water and cultured on Medium III. Two months after spindle toxin treatment, the number of apical segments with elongated shoots (over 1.5 cm in length) were recorded.

Flow cytometry (FCM) analysis

Ploidy levels of apical segment-derived shoots were estimated by FCM analysis of leaf tissues using a flow cytometer PA (Partec, GmbH-Münster, Germany) as previously described (Saito *et al*., 2003). At least 1,500 nuclei were examined for each shoot.

RESULT AND DISCUTTION

Four months after culture on Medium III, multiple shoots consisting of more than 30 shoots were obtained from two out of 11 shoot tips (Fig. 1). Apical segments of these proliferated shoots were used for spindle toxin treatment.

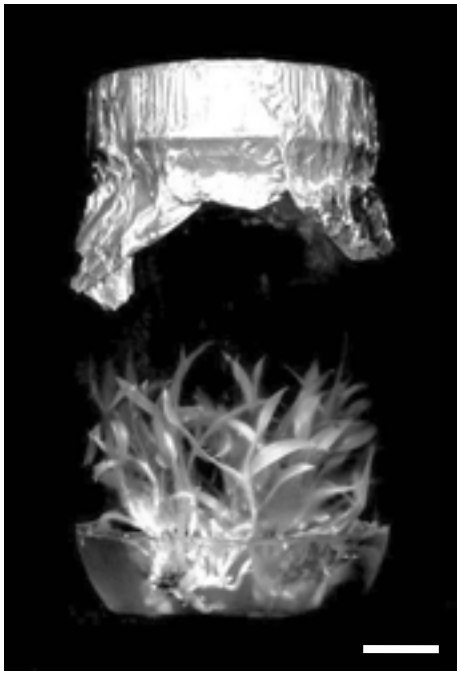

Fig 1. Shoot tip-derived multiple shoots of an intergeneric hybrid between *Littonia modesta* and *Gloriosa superba* 'Lutea' four months after culture on Medium III. Bar = 2 cm.

Table 1 shows effect of spindle toxin treatments of apical segments on elongation of apical segment-derived shoots and ploidy levels of apical segment-derived shoots 2 months after spindle toxin treatment. After spindle toxin treatment, some apical segments turned brown and died, but some segments survived and developed shoots. In all treatments including the control one, each apical segment developed only one shoot. In the control treatment, 56.7% of apical segments elongated shoots. There are no significant differences in the percentage of apical segments with shoot elongation among the control and three spindle toxin treatments, 10 mg L^{-1} APM , 100 mg L^{-1} COL and 500 mg L^{-1} COL. The highest percentage of apical segments shoot elongation (80.0%) was obtained in 10 mg L^{-1} APM treatment.

In order to estimate the ploidy level of spindle toxin treatment-derived shoots, FCM analysis of leaf tissues was performed (Fig. 2). In the diploid mother shoots (2*x*), histogram showed a single peak corresponding to nuclei in the G0/G1 phase of the cell cycle, and neither ploidy chimera nor polysomaty were found. The G0/G1 peak of all the 17 shoots, which were derived from the control treatment, appeared at almost same position as the mother shoots, indicating that they were diploid (2*x*). On the other hand, a single G0/G1 peak corresponding to tetraploid (4*x*) appeared in a histogram of one shoot derived from 40 mg L^{-1} ORY treatment. In some shoots derived from treatments with 10 or 20 mg L^{-1} APM or 5 or 10 mg L^{-1} ORY, histograms showed two G0/G1 peaks appeared at different positions, indicating

Table 1. Effect of spindle toxin treatments of apical segments on elongation of apical segment-derived shoots and ploidy levels of apical segment-derived shoots in an intergeneric hybrid between *Littonia modesta* and *Gloriosa superba* 'Lutea'.

Spindle toxin	Concentration (mg L^{-1})	No. of apical segments treated	No. of apical segments with shoot elongation [a]	% of apical segments with shoot elongation [b]	No. of apical segment-derived shoots of each ploidy level [c]			% of tetraploid and ploidy chimera shoots
					Diploid (2*x*)	Tetraploid (4*x*)	Ploidy chimera (2*x*+4*x*)	
Control [d]	–	30	17	56.7 a	17	0	0	0
APM	10	30	24	80.0 a	18	0	6	25.0
	20	30	10	33.3 b	7	0	3	30.0
	40	30	3	10.0 b	2	1	0	33.3
COL	100	30	14	46.7 ab	14	0	0	0
	500	30	13	43.3 ab	13	0	0	0
	1000	30	10	33.3 b	10	0	0	0
ORY	5	30	6	20.0 b	5	0	1	16.7
	10	30	6	20.0 b	4	0	2	33.3
	20	30	1	3.3 c	1	0	0	0

[a] Apical segments with elongated shoots (over 1.5 cm in length) were recorded two months after spindle toxin treatment.
[b] Values represent the mean of three independent experiments each of which consisted of 10 apical segments. Means followed by the same letter are not significantly different at the 0.05 level with Ryan's test.
[c] Ploidy level was determined by FCM analysis using leaf tissues of apical segment-derived shoots.
[d] Apical segments were treated with liquid Medium III without any spindle toxins.

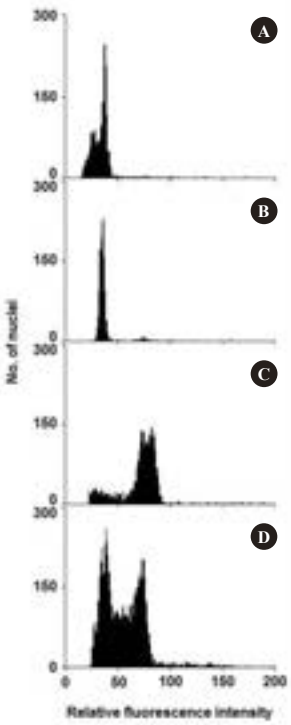

Fig 2. Histograms from FCM analysis of nuclear DNA content of shoots derived from spindle toxin-treated apical segments in an intergeneric hybrid between *Littonia modesta* and *Gloriosa superba* 'Lutea'. **A**, Diploid mother shoot (2*x*); **B**, diploid shoot (2*x*) derived from the control treatment; **C**, tetraploid shoot (4*x*) derived from 40 mg L^{-1} APM treatment; **D**, ploidy chimera shoot (2*x*+4*x*) derived from 10 mg L^{-1} APM treatment.

that they were ploidy chimera (2*x*+4*x*). The highest percentage of chromosome-doubled shoots (33.3%) was obtained in treatments with 40 mg L^{-1} APM or 10 mg L^{-1} ORY (Table 1). In COL treatments, neither tetraploid nor ploidy chimera were obtained. These results indicate that the kind and concentration of spindle toxins affected shoot elongation and chromosome doubling in an intergeneric hybrid between *L. modesta* and *G. superba* 'Lutea', and 10 mg L^{-1} APM treatment effectively induced chromosome doubling with a little damage to apical segments. It has bee reported that APM and/or ORY are more effective than COL for inducing chromosome doubling in several ornamental plants (Thao *et al.*, 2003; Nonaka *et al.*, 2011). Furthermore, Lit × Gsu-1 might has some resistance to COL, since colchicaceous plants naturally produce COL (Larsson *et al.*, 2004).

Tetraploid and ploidy chimera shoots obtained in the present study showed compact forms with reduced internodes compared with diploid shoots (Fig. 3). Similar observations have been reported in chromosome-doubled plants of several plant species (Nonaka *et al.*, 2011; Trojak-Goluch and Skomra, 2013). Compact plant forms may be valuable in colchicaceous ornamental plants especially for a pot use.

Tetraploid and ploidy chimera shoots were transferred to MS medium without plant growth regulators for root induction. However, all of them died without root formation, although some ploidy chimera shoots produced tuber-like structures. Therefore, further studies are necessary to induce root formation of chromosome-doubled shoots in colchicaceous ornamental plants.

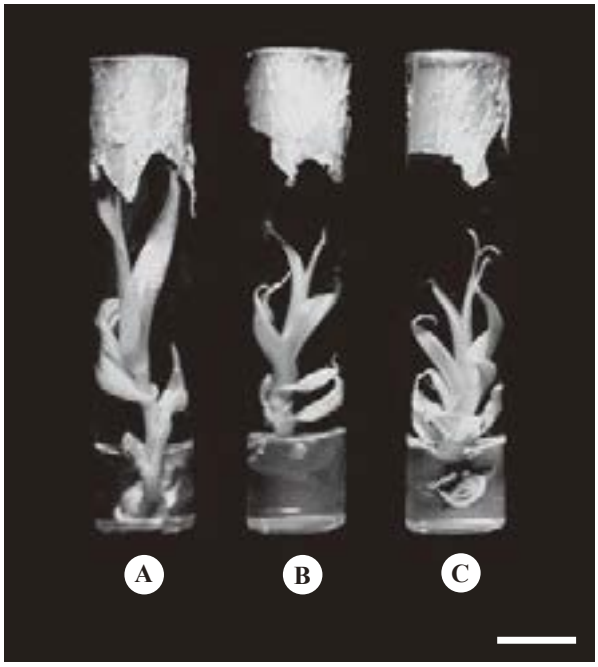

Fig 3. Shoots of an intergeneric hybrid between *Littonia modesta* and *Gloriosa superba* 'Lutea' derived from spindle toxin-treated apical segments. **A**, Diploid shoot (2*x*) derived from the control treatment; **B**, tetraploid shoot (4*x*) derived from 40 mg L^{-1} APM treatment; **C**, ploidy chimera shoot (2*x*+4*x*) derived from 10 mg L^{-1} APM treatment. Bar = 1 cm.

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紡錘糸形成阻害剤処理によるコルチカム科属間雑種の染色体倍加

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要　約

コルチカム科には、グロリオーサやサンダーソニアなど、重要な花き園芸植物が含まれている。我々は、園芸形質の拡大および新奇品種の育成のために、これまでコルチカム科内で様々な属間雑種を作出してきた。本研究では、稔性の回復および更なる園芸形質の拡大を目的として、コルチカム科の二倍体属間雑種であるリットニア × グロリオーサ‘ルテア’（*Littonia modesta* × *Gloriosa superba*‘Lutea’；Lit × Gsu-1；$2n=2x=22$）の染色体倍加を試みた。シュート増殖培養物のシュートから茎頂を含む切片を調製し、様々な濃度のアミプロホスメチル（APM；10、20 または 40 mg L-1）、オリザリン（ORY；5、10 または 20 mg L^{-1}）またはコルヒチン（COL；100、500 または 1000 mg L^{-1}）を添加した液体培地で 24 時間振とう処理した。紡錘糸形成阻害剤処理２ヵ月後、10 mg L^{-1} APM 処理区において最も効率的に茎頂切片からシュートが伸長した。伸長したシュートの葉組織を用いてフローサイトメトリー分析を行ったところ、40 mg L^{-1} APM 処理区で四倍体（$4x$）のシュートが、また、10 または 20 mg L^{-1} APM および 5 または 10 mg L^{-1} ORY 試験区で倍数性キメラ（$2x+4x$）のシュートが確認された。これらの四倍体および倍数性キメラのシュートは、二倍体のシュートと比較して、節間が短縮したコンパクトな草姿を示した。

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