Chromosome Doubling of Japanese Native *Lychnis* spp. by *in vitro* Oryzalin Treatment

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Summary

The genus *Lychnis* (Caryophyllaceae) consists of about 30 species, among which 6 are native to Japan. Many *Lychnis* spp. are cultivated as pot or garden plants for their high ornamental value. In the present study, chromosome doubling of 3 Japanese native *Lychnis* spp., *L. gracillima* (2*n*=2*x*=24), *L. kiusiana* (2*n*=2*x*=24) and *L. miqueliana* (2*n*=2*x*=24), was examined for widening their variability in horticultural traits by using an *in vitro* oryzalin (ORY) treatment method, which was initially developed for a triploid genotype of *L. senno* (Nonaka *et al.,* 2011). Nodal segments prepared from *in vitro*-grown plantlets of these 3 species were treated with 10 mg L^{-1} ORY for 24 h. Two months after ORY treatment, more than 70% of nodal segments survived in all the 3 *Lychnis* spp. Flow cytometry analysis of leaf tissues showed that 15.7, 15.1 and 7.8% of plantlets, which were derived from ORY-treated nodal segments, were tetraploid (4*x*) or ploidy chimera (2*x*+4*x*) in *L. gracillima, L. kiusiana* and *L. miqueliana,* respectively. Chromosome doubling was also confirmed by chromosome observation in root-tip cells. These results indicate that *in vitro* ORY treatment may be applicable for chromosome doubling of a wide range of *Lychnis* spp. Tetraploid plantlets obtained in the present study showed a compact plant form compared with diploid plantlets.

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Key words : caryophyllaceous ornamental, *Lychnis gracillima, L. kiusiana, L. miqueliana,* tetraploid

The genus *Lychnis*, a member of Caryophyllaceae, consists of about 30 species, which are distributed throughout the temperate regions of the Northern Hemisphere (Magnus *et al.,* 2008). Among them, 6 species, *L. fulgens, L. gracillima, L. kiusiana, L. miqueliana, L. sieboldii* and *L. wilfordii,* are native to Japan. Most *Lychnis* spp., including Japanese native ones, are cultivated as pot and garden plants for their beautiful flowers. However, all the species have few variations in horticultural traits, such as flower color, flower form and plant form.

Previously we examined effect of *in vitro* spindle toxin treatment on chromosome doubling of a triploid genotype of *L. senno* in order to widen its variability in horticultural traits (Nonaka *et al.,* 2011). Efficient chromosome doubling was achieved by treating nodal segments with 10 mg L^{-1} oryzalin (ORY), and this method was successfully applied to diploid genotypes of *L. fulgens* and *L. sieboldii* (Nonaka *et al.,* 2011).

In the present study, we examined chromosome doubling of 3 Japanese native *Lychnis* spp., *L. gracillima, L. kiusiana* and *L. miqueliana,* by using the *in vitro* oryzalin treatment method developed by Nonaka *et al.* (2011). Chromosome doubling of plantlets derived from ORY-treated nodal segments was confirmed by both flow cytometry (FCM) analysis and chromosome observation.

MATERIALS AND METHODS

Plant materials and shoot culture

L. gracillima (2*n*=2*x*=24), *L. kiusiana* (2*n*=2*x*=24) and *L. miqueliana* (2*n*=2*x*=24) were used in the present study. Potted plants were cultivated in the greenhouse without heating at the Faculty of Agriculture, Niigata University.

Shoot cultures of 3 *Lychnis* spp. were established according to Nonaka *et al.* (2011). Briefly, nodal explants were prepared from vigorously growing stems of potted plants and placed on a shoot culture medium [Murashige and Skoog (1962) (MS) medium supplemented with 10 mg L^{-1} benzyl adenine, 30 g L^{-1} sucrose and 2 g L^{-1} gellan gum, pH 5.7]. Axillary bud-derived shoots were subcultured every 2 months by transferring nodal segments to fresh medium of the same composition. Cultures were maintained at 25℃ under continuous illumination with fluorescent light (35 μmol m^{-2} s^{-1}).

ORY treatment and plant regeneration

In vitro ORY treatment of nodal segments was performed according to Nonaka *et al.* (2011). Liquid shoot culture medium supplemented with 10 mg L^{-1} ORY was used as an ORY treatment solution. ORY was purchased from AccuStandard Inc. (USA). Liquid shoot culture medium without ORY was used as a control. Nodal segments (ca. 10 mm in length) were harvested from shoot cultures 2 months after subculture. After removing leaves, nodal segments were

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soaked in the ORY treatment solution and incubated on a rotary shaker (100 rpm) at 25℃ for 24 h. Nodal segments were rinsed 3 times with sterile, distilled water and placed on the shoot culture medium. Cultures were maintained at 25℃ under continuous illumination with fluorescent light (35 μmol m^{-2} s^{-1}). Number of surviving nodal segments, number of elongating shoots from nodal segments, and length of elongating shoots were recorded 2 months after ORY treatment.

Within 3 months after ORY treatment, nodal segment-derived shoots elongating over 1.5 cm in length were excised from the nodal segments and transferred to rooting medium (half-strength MS medium supplemented with 30 g L^{-1} sucrose and 2 g L^{-1} gellan gum, pH 5.7). Cultures were maintained at 25℃ under continuous illumination (35 μmol m^{-2} s^{-1}).

FCM analysis and chromosome observation

Ploidy level of ORY 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 according to Nakamura *et al.* (2005).

RESULTS AND DISCUSSION

Table 1 shows effect of ORY treatment on survival and development of nodal segments. In the control treatment without ORY, all the nodal segments survived in both *L. gracillima* and *L. kiusiana*, whereas 84.0% of nodal segments did in *L. miqueliana*. Genotypic differences in the number of shoots per surviving nodal segments and mean length of shoots were observed: shoot number and length were much higher in *L. kiusiana* and *L. gracillima*, respectively. After ORY treatment, some nodal segments turned brown and died, but most segments survived and developed shoots from axillary buds within 1 month after ORY treatment. Although the percentage of surviving segments in ORY treatment was slightly lower than in the control, more than 70% of ORY-treated segments survived in all the 3 *Lychnis* spp. No large differences in the number and growth of nodal segment-derived shoots were observed between the control and ORY treatment in all the 3 *Lychnis* spp.

Some nodal segment-derived shoots that elongated over 1.5 cm in length were randomly selected and transferred to the rooting medium, on which almost all the shoots produced roots and developed into plantlets. In order to estimate the ploidy level of ORY treatment-derived plantlets, FCM analysis of leaf tissues was performed (Fig. 1). In all the 3 *Lychnis* species, the diploid mother plantlets showed histograms with 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 plantlets, which were derived from the control treatment, appeared at almost the same position as the mother plantlets, indicating that they were diploid (Table 2). On the other hand, G0/G1 peak corresponding to tetraploid ($4x$) appeared in histograms of ORY treatment-derived plantlets. In several ORY treatment-derived plantlets, 2 G0/G1 peaks appeared at different positions, indicating that they were ploidy chimera ($2x$+$4x$). In *L. gracillima*, *L. kiusiana* and *L. miqueliana*, 15.7, 15.1 and 7.8% of ORY treatment-derived plantlets, respectively, were tetraploid or ploidy chimera. More than 12% of ORY treatment-derived plantlets were solid diploid in both *L. gracillima* and *L. kiusiana*. The ploidy level of ploidy chimeras ($2x$+$4x$) of all the 3 *Lychnis* spp. was unstable, and they tended to become solid diploids or tetraploids after several subcultures.

The results obtained by FCM analysis was confirmed by chromosome counting in root-tip cells (Fig. 2). *L. gracillima* plantlets estimated to be solid tetraploid by FCM analysis had $2n$=48 chromosomes. A similar observation was also obtained in both *L. kiusiana* and *L. miqueliana* (data not shown).

In all the 3 *Lychnis* spp., solid tetraploid plantlets and ploidy chimera plantlets with high proportion of tetraploid cells showed a compact plant form and had deep green leaves compared with the diploid mother plantlets (Fig. 3).

Table 1. Effect of ORY treatment of nodal segments on survival of nodal segments and production of nodal segment-derived shoots in 3 Japanese native *Lychnis* spp.[a]

Lychnis spp.	ORY concentration (mg L^{-1})	No. of segments treated	No. of surviving segments	% of surviving segments	No. of shoots per surviving segment[b]	Shoot length (mm)[b]
L. gracillima	0	25	25	100	2.6 ± 0.1	24.4 ± 0.3
	10	66	55	83.3	2.2 ± 0.1	24.2 ± 0.2
L. kiusiana	0	25	25	100	20.0 ± 0.2	5.0 ± 0.1
	10	60	54	90.0	20.3 ± 0.6	5.2 ± 0.1
L. miqueliana	0	25	21	84.0	2.3 ± 0.2	6.1 ± 0.1
	10	50	37	72.0	1.5 ± 0.1	5.4 ± 0.1

[a] Data were recorded 2 months after ORY treatment.

[b] Values represent the mean ± standard error.

Table 2. Effect of ORY treatment of nodal segments on the ploidy level of nodal segment-derived plantlets in 3 Japanese native *Lychnis* spp.

Lychnis spp.	ORY concentration (mg L^{-1})	No. of plantlets analyzed	No. of plantlets of each ploidy level [a]			% of tetraploid + ploidy chimera plantlets
			Diploid (2*x*)	Tetraploid (4*x*)	Ploidy chimera (2*x*+4*x*)	
L. gracillima	0	60	60	0	0	0
	10	108	91	13	4	15.7
L. kiusiana	0	49	49	0	0	0
	10	106	94	13	3	15.1
L. miqueliana	0	43	43	0	0	0
	10	51	47	3	1	7.8

[a] Ploidy level was determined by FCM analysis using upper 3 leaves of each plantlet 4 months after ORY treatment.

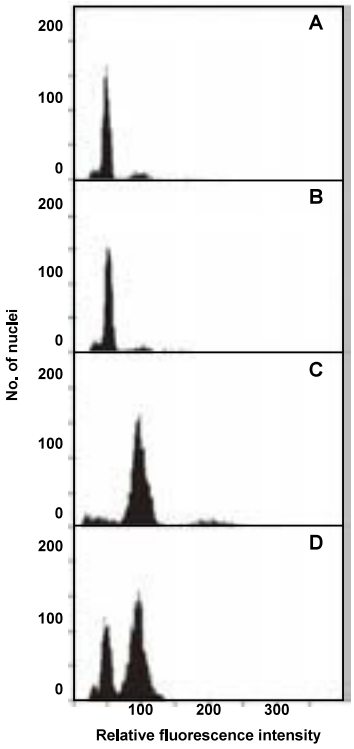

Fig. 1. Histograms from FCM analysis of nuclear DNA content of *Lychnis kiusiana* plantlets derived from ORY-treated nodal segments. **A**, Diploid mother plant (2*x*); **B**, diploid (2*x*) derived from the control treatment without ORY; **C**, tetraploid (4*x*) derived from 10 mg L^{-1} ORY treatment; **D**, ploidy chimera (2*x*+4*x*) derived from 10 mg L^{-1} ORY treatment.

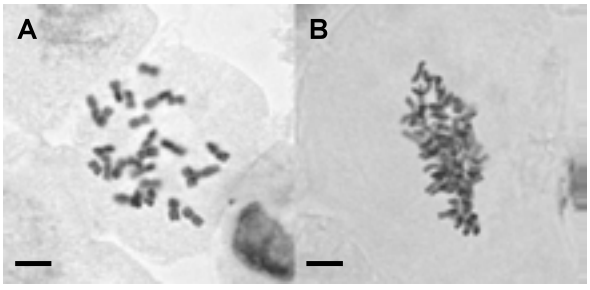

Fig. 2. Somatic chromosomes in root-tip cells of *Lychnis gracillima* plantlets derived from ORY-treated nodal segments. **A**, Diploid mother plant (2*n*=2*x*=24); **B**, tetraploid (2*n*=4*x*=48) derived from 10 mg L^{-1} ORY treatment. Each bar represents 1 μm.

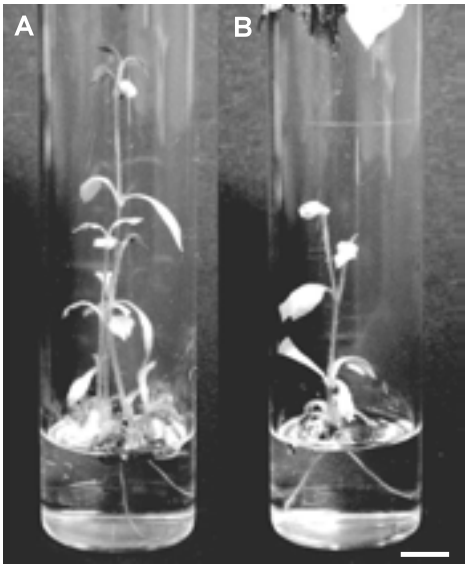

Fig. 3. *Lychnis kiusiana* plantlets derived from ORY-treated nodal segments. **A**, Diploid mother plant (2*x*); **B**, tetraploid (4*x*) derived from 10 mg L^{-1} ORY treatment. Bar represents 1 cm.

Similar observation has also been reported for chromosome-doubled plantlets of triploid *L. senno* (Nonaka *et al.,* 2011).

In vitro treatment of nodal segments with 10 mg L^{-1} ORY was initially developed for a triploid genotype of *L. senno*, in which 75.0% of nodal segments survived after ORY treatment and 18.8% of ORY treatment-derived plantlets were hexaploid or ploidy chimera (Nonaka *et al.,* 2011). In the present study, application of 10 mg L^{-1} ORY treatment to 3 Japanese native *Lychnis* spp., *L. gracillima*, *L. kiusiana* and *L. miqueliana*, yielded comparable percentages of surviving nodal segments (72.0–90.0%) and tetraploid or ploidy chimera plantlets (7.8–15.7%), indicating that this treatment may be applicable for chromosome doubling of a wide range of *Lychnis* spp.

Chromosome doubling has been used as a breeding tool in various ornamental plants for widening the variability in horticultural traits (Väinölä, 2000). Polyploidization generally leads to larger flowers, compact growth habit, thicker stems, deeper green leaves or increased width-to-length ratio of leaves (Chen and Goeden-Kallemeyn, 1979; Lindsay *et al.,* 1994; Gao *et al.,* 1996; Takamura and Miyajima, 1996; Nakano *et al.,* 2006; Tang *et al.,* 2010). Tetraploid plants of *L. fulgens* and *L. sieboldii,* which were produced by *in vitro* ORY treatment, showed a compact plant form, thick stems and deep green leaves compared with the diploid mother plants (Nonaka *et al.,* 2011). Such morphological alterations are attractive in a breeding program of *Lychnis* spp. Therefore, further studies should be directed to cultivation and morphological characterization of tetraploid plants produced in the present study.

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培養物へのオリザリン処理による日本原産センノウ属植物の染色体倍加

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要　約

センノウ属（*Lychnis* spp.；ナデシコ科）は約30種で構成され、そのうちの６種は日本に自生している。多くのセンノウ属植物は観賞的価値が高く、そのため鉢植え植物や庭植え植物として栽培されている。本研究では、園芸形質の拡大を目的として、日本原産の３種のセンノウ属植物、センジュガンピ（*L. gracillima*；2*n*=2*x*=24）、オグラセンノウ（*L. kiusiana*；2*n*=2*x*=24）およびフシグロセンノウ（*L. miqueliana*；2*n*=2*x*=24）について、培養物のオリザリン（ORY）処理による染色体倍加を試みた。これは、三倍体のセンノウ（*L. senno*）において最初に確立された方法である（Nonaka ら、2011）。培養小植物体から調製した節切片を10 mg L^{-1} ORY で24時間処理したところ、２ヵ月後にはいずれのセンノウ属植物においても70% 以上の節切片が生存していた。また、ORY 処理した節切片由来の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、センジュガンピ、オグラセンノウおよびフシグロセンノウの小植物体のそれぞれ15.7、15.1および7.8% が四倍体（4*x*）または倍数性キメラ（2*x*+4*x*）であった。染色体倍加は、根端細胞の染色体観察によっても確認された。以上の結果は、培養物への ORY 処理が、広範囲のセンノウ属植物における染色体倍加に適用できることを示している。本研究で得られた四倍体小植物体は、二倍体小植物体と比較して、草姿が小型であった。

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キーワード：オグラセンノウ、センジュガンピ、ナデシコ科観賞植物、フシグロセンノウ、四倍体

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