Morphological and Physiological Changes in Transgenic *Chrysanthemum morifolium* Ramat. ‘Ogura-nishiki’ with *rolC*

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The effects of *rolC* on morphogenesis of *Chrysanthemum morifolium* Ramat. ‘Ogura-nishiki’ were studied. The ray flowerets in the *rolC*-transformed plants were shorter than those in wild-type plants, but the transformation enhanced outgrowth of lateral shoots and reduced internode elongation. Studies on adventitious shoot and root formation from leaf segments revealed that the *rolC* transgene led to reduced root formation but enhanced adventitious bud formation. This enhanced cytokinin-like activity was consistent with phenotypes observed in the *rolC*-transformed plants.

Key Words: chrysanthemum, *rolC*, transformation.

Introduction

The soil bacterium *Agrobacterium rhizogenes* causes hairy root disease in a range of dicotyledonous plants. The *rolA*, *rolB*, *rolC*, and *rolD* genes have a central role in the bacterium’s ability to incite hairy roots (White et al., 1985). Schmülling et al. (1988) who investigated the biological effects of these genes in transgenic tobacco showed that overexpression of *rolC* decreased internode elongation and increased the number of shoots. Such alterations have also been reported for other *rolC*-transformed species such as potato (Fladung, 1990; Schmülling et al., 1993), belladonna (Kurioka et al., 1992), hybrid aspen (Nilsson et al., 1996) and carnation (Zuker et al., 2001). Moreover, *rolC* affects the morphogenesis of plant tissues; Casanova et al. (2003) showed that *rolC* enhanced adventitious shoot and root formation from carnation leaves.

A number of transgenic chrysanthemum plants have been produced by using *Agrobacterium*-mediated transformation techniques (De Jong et al., 1994; Fukai et al., 1995; Ledger et al., 1991; Renou et al., 1993; Sherman et al., 1998a; Urban et al., 1994; Wordragen et al., 1991). Some genes that code practical

characteristics such as TSWV nucleocapsid gene (Sherman et al., 1998b), rice chitinase gene (Takatsu et al., 1999), BT gene (Shinoyama et al., 2002), phytochrome *B1* gene (Zheng et al., 2001), *Arabidopsis gai* gene (Petty et al., 2003) and Rice *OsMADS1* gene (Jeong et al., 2002) have also been introduced to chrysanthemum.

The dwarfed, bushy phenotype, observed in *rolC*-transformed plants, is preferred for potted flower plants. Chrysanthemums, transformed with *rolC* which has the dwarfed, bush habit, have already been produced by Mitouchkina and Dolgov (2000), but, these plants also had smaller flowers. The *rolC* gene introduced in their study was driven by CaMV 35S promoter, which was effective throughout the plant. The *rolC* expression, driven by *rolC* native promoter, was high in root and stem, but low in leaves (Nakamura et al., 1988). This indicates that the chrysanthemums, transformed with *rolC* under *rolC* native promoter, may have the dwarfed, bushy phenotype with normal size flowers.

In this study, we transformed chrysanthemum with *rolC* under *rolC* native promoter and observed the resultant transgenic plants to evaluate the horticultural effect of this particular gene. Furthermore, adventitious shoot and root formation from leaves was investigated in *rolC*-transformed chrysanthemums to consider the physiological effect of *rolC*.

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Materials and Methods

Plant material

Chrysanthemum morifolium Ramat. ‘Ogura-nishiki’, which belongs to the Japanese chrysanthemum group ‘Sagagiku’ was used as the plant material. This cultivar shows a high regenerate ability (more than 90% of leaf segments were regenerated on MS medium, containing 5.4×10^{-7} M NAA and 8.8×10^{-6} M BA) (Tsuro et al., 2002). The aseptic shoots, cultured on 1/2 Murashige and Skoog (1962) (MS) medium (half inorganic salt concentration), were used for transformation.

Plant transformation

Disarmed *Agrobacterium tumefaciens* LBA4404 harboring a construct prepared by replacing the sequence of the CaMV 35S promoter and β -glucuronidase gene for that of the *rolC* gene with an original promoter derived from pRi1724 (Handa et al., 1995) was used for transformation. Transformation was performed according to the method of Tsuro et al. (2005). Putative transformants were screened for the presence of *rolC* genes by PCR analysis. Genomic DNA was extracted from leaf tissue by a modified cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich, 1985). PCR was conducted by using the oligonucleotide primer pair (*rolC1* and *rolC2*) specific for *rolC*; *rolC1* (5'-TGATGCGATGCTTTTATGGA-3') and *rolC2* (5'-GGCATAAAGGTCTGAAGGTCA-3') primers amplify a 330 bp fragment. Thirty-five PCR cycles were performed in a thermal cycler (Applied Biosystem 9600, USA), by using a step-cycle program of 94°C for 30 s, 57°C for 30 s and 73°C for 30 s.

Investigation of morphological characteristics

Two lines of transformants which showed significant morphological changes in vitro were investigated. A line of non-transformed in vitro plantlets was used as control line. Rooted plants about 3 cm in height were removed from the medium, and their roots were cleaned of gelfrite. They were then transferred to plastic pots (15 cm in diameter), filled with water-saturated Metro Mix (Scotts, USA), the plants were kept under high humidity and at 25°C. After 2 weeks of acclimatization, 5 plants from each line were grown in a greenhouse maintained at 25°C under natural daylight in early March.

Twelve weeks after completion of acclimatization, the number of shoots, length of the longest shoots, and average internode length between the 5th and 10th nodes were recorded. Then, plants were grown under an 11-h photoperiod by shading with a black sheet to induce inflorescence in early August. The numbers of ray and tubular florets were counted from a random sample of 10 inflorescences (per line) and the lengths of five ray and tubular florets per inflorescence were measured. Means for transgenic and control lines were separated by using the *t*-test ($P < 0.05$).

RT-PCR analysis for *rolC* expression

Total RNAs were extracted from leaves and roots of wild-type and transformed plants by a modified CTAB method (Chang et al., 1993) and treated with DNase to eliminate any DNA contamination. cDNA was synthesized from 4 μ g of total RNA with a cDNA synthesizer kit (Amersham Bioscience, USA). cDNA synthesis reaction was induced under 37°C for 1 h. Expression of *rolC* was detected by RT-PCR by using the same primers as for detecting the transgene. Thirty PCR cycles were performed in a thermal cycler (Applied Biosystem 9600), by using a step-cycle program of 94°C for 30 s, 57°C for 30 s and 73°C for 30 s.

Adventitious root and shoot formation from leaf explants

Leaves from the in vitro plants of wild-type and *rolC*-introduced were cut into 5 $\times$ 5 mm segments. Sixteen explants were placed on MS medium (3% sucrose and 0.3% gelfrite) containing 5.4×10^{-7} M NAA for root regeneration or 5.4×10^{-7} M NAA and 2.2×10^{-6} M BA for shoot regeneration in 9-cm plastic plates and cultured in the dark at 25°C. Five plates were used for each line. Root and shoot regeneration were investigated 2 and 4 weeks after culture, respectively. Means for transgenic and control lines were separated by using the *t*-test ($P < 0.05$).

Results

Morphological characteristics of *rolC*-transformed plants

A total of 1008 leaf segments were used for transformation, from which 23 shoots were obtained as putative transformants. Fourteen of them were confirmed as being transformants by PCR analysis (Fig. 1). The frequency of transformed plants was about 1.4%. As observed in in vitro plantlets, all transformants exhibited enhanced branching and some with shortened internodes. Two lines that showed dwarf obviously, *oprolC*-17 and

Fig. 1. Gel plate for PCR analysis of transgenic chrysanthemums ('Ogura-nishiki'). Lane M, Size marker derived from ϕ X174/*Hae*III; Lane P, positive control from *A. tumefaciens* used for transformation; Lanes *oprolC*, putative transformants regenerated on the medium containing 100 mg·L⁻¹ kanamycin; Lane N, non-transformant.

Fig. 2. Gel plates for RT-PCR analysis of *rolC*-transformed chrysanthemums. Total RNAs were extracted from leaves (lanes S) and roots (lanes R) of wild-type, oprolC-17 and -18 plants grown in a glasshouse and used for cDNA synthesis. Lane M was size marker derived from ϕ X174/*Hae*III.

-18, were investigated morphologically.

rolC expressions in these transformants were detected in the leaves and roots of both lines (Fig. 2). Morphological differences between the wild-type and *rolC*-transgenic plants (Fig. 3) showed enhanced branching of both oprolC-17 and -18: the numbers of shoots were 4.2 and 3.8, respectively, compared with 1.0 in the wild-type (Table 1). Furthermore, *rolC*-transgenic plants had shortened internodes: shoot lengths of oprolC-17 and -18 were 63.4 and 39.8% of the wild-type, respectively.

Time until flowering was similar in *rolC*-transgenic and wild-type plants; about 8 weeks after starting the short day treatment. The numbers of ray and tubular florets per inflorescence were not significantly different between the oprolC-17 and -18 plants and the wild-type plants (Table 2); but petal lengths of ray florets in the oprolC-17 and -18 plants were shorter than those of the wild-type plants (Table 2, Fig. 4).

Adventitious root and shoot formation from leaf segments of *rolC*-transformed plants

Adventitious root formation from leaf segments was reduced in oprolC-17 and -18. Two weeks after the culture, the percentages of rooting explants were 35.0 ± 5.8 and $26.3 \pm 4.1\%$ in oprolC-17 and -18, respectively, in comparison with $88.8 \pm 4.1\%$ in the wild-type (Fig. 5), and the numbers of roots per regenerated explant were 1.9 and 1.7, respectively, as compared to 4.3 in the wild-type.

Leaf segments in oprolC-17 and -18 formed more adventitious shoots. Four weeks after the culture, the percentages of adventitious shoot formation explants were 26.3 ± 7.6 and $31.3 \pm 3.5\%$ in oprolC-17 and -18, respectively, in comparison with $6.3 \pm 2.5\%$ in the wild-type (Fig. 5).

Wild-type oprolC-17 oprolC-18

Fig. 3. Morphological differences between a wild-type and *rolC*-transformed plants grown in a greenhouse kept at 25°C for 8 weeks.

Table 1. Number of shoots, and shoot and internode lengths of wild-type and transformants 12 weeks after being transferred to a greenhouse.

	Number of shoots ^z	Shoot length ^y (cm)	Internode length ^x (cm)
Wild-type	1.0 ± 0^w	39.9 ± 0.6	2.4 ± 0.1
oprolC-17	$4.2 \pm 0.2^*$	$25.3 \pm 2.1^*$	$1.3 \pm 0.1^*$
oprolC-18	$3.8 \pm 0.2^*$	$15.9 \pm 2.2^*$	$0.8 \pm 0.2^*$

* indicates significant differences between *rolC*-transgenic and wild-type lines according to the *t*-test ($P < 0.05$).

^z Numbers of shoots over 5 cm were counted.

^y Shoot lengths of the longest shoots were measured.

^x Average values of the fifth to tenth leaves.

^w Means $\pm$ SE ($n = 5$).

Discussion

The *rolC* gene under *rolC* native promoter induced more lateral shoots, while reducing the internode lengths

Table 2. Florets number and length of wild-type and transformants.

	Floret number per inflorescence ^z		Floret length (cm) ^y	
	Tubular florets	Ray florets	Tubular florets	Ray florets
Wild-type	68.5 ± 6.9 ^x	49.8 ± 6.1	0.73 ± 0.08	3.0 ± 0.2
oprolC-17	75.8 ± 9.6	43.2 ± 8.2	0.71 ± 0.13	2.0 ± 0.3*
oprolC-18	59.0 ± 9.6	43.3 ± 6.6	0.79 ± 0.12	1.7 ± 0.2*

* indicates significant differences between *rolC*-transgenic and wild-type lines according to the *t*-test ($P < 0.05$).

^z The numbers of ray and tubular florets were counted from a random sample of 10 inflorescences.

^y The length of each five of ray and tubular florets per inflorescence were measured.

^x Means ± SE (n = 10).

Wild-type *rolC*-transformants

Fig. 4. Morphological differences between inflorescences of a wild-type and *rolC*-transformant.

in chrysanthemum. The effects were similar to the effects of the *rolC* gene under CaMV 35S promoter (Mitiouchkina and Dolgov, 2000). The purpose in this study was the production of *rolC*-transformed chrysanthemum with a bush phenotype but normal size flowers. But, this was not achieved as the chrysanthemums transformed with *rolC* under *rolC* native promoter had smaller size flower. Malformed leaves and flowers by introduction of the *rolC* gene under CaMV 35S promoter have been reported in chrysanthemum (Mitiouchkina and Dolgov, 2000), but these malformations were not detected in the chrysanthemum transformed with *rolC*

under *rolC* native promoter. This promoter difference seems to have been involved in the different morphological changes. But, only 2 lines were investigated in this study; another study by Handa (1992) reported malformed leaves and flowers in plants transformed by the *rolC* gene under *rolC* native promoter. Hence, further studies are required.

The *rolC* transgene led to reduced adventitious root formation. Furthermore *rolC*-transformed chrysanthemums could regenerate from leaf segment on the medium that contains even a low concentration of cytokinin (Fig. 5). Improved shoot regeneration was also observed in *rolC*-transformed carnations (Casanova et al., 2003). This enhanced cytokinin-like activity is consistent with the *rolC*-induced phenotypes observed in our *rolC*-transformed chrysanthemum plants; that is enhanced outgrowth of lateral shoots and reduced elongation of internodes. Estruch et al. (1991) showed that *rolC* codes for a cytosolic β -glycosidase release free forms of cytokinin from their inactive glucosidic conjugates. The expression of *rolC* might therefore change cytokinin levels as a secondary effect during hormone metabolism.

This study showed that the introduction of *rolC* under *rolC* native promoter to chrysanthemums has advantageous effects in potted flower plants by enhancing outgrowth of lateral shoots and reducing internode elongation. However, it was simultaneously disadvantageous in that it reduced flower size.

Fig. 5. Effect of transformation with *rolC* on adventitious root (A) and shoot (B) formation from leaf explants. Values are means ± SE (n = 5).

* indicates significant differences between *rolC*-transgenic and wild-type lines according to the *t*-test ($P < 0.05$).

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rolC 遺伝子導入による嵯峨ギク ‘小倉錦’ の形態および生理的变化

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rolC 遺伝子の導入が嵯峨ギク ‘小倉錦’ の形態形成に及ぼす影響について調査したところ、*rolC* 遺伝子導入により、舌状花長の減少が認められたものの、多分枝およびわい性といった鉢物に適した形質を付与できることが示された。また、葉片からの不定芽および不定根形成に

ついて調査したところ、*rolC* 遺伝子導入による不定根形成の抑制および不定芽形成の促進が認められた。これらの形態および生理的影響はサイトカイニンの作用に類似しており、*rolC* 遺伝子導入による内生サイトカイニン量の変化が示唆された。