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Original Paper

Production of dwarf potted gentian using wild-type *Agrobacterium rhizogenes*

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Abstract We successfully produced dwarf potted gentian using wild-type *Agrobacterium rhizogenes* A4 strain (ATCC43057) harboring an agropine type plasmid (pRiA4). A number of hairy roots were induced by direct inoculation with *A. rhizogenes* to the stem and leaf tissues *in vitro*. Adventitious shoots were regenerated from the hairy roots on Murashige and Skoog medium containing 10 mg l^{-1} N-phenyl-N′-(1,2,3-thiadiazol-5-yl) urea, 0.1 or 1.0 mg l^{-1} 1-naphthaleneacetic acid, and 3 g l^{-1} gellan gum. Regenerated plants were then cultured *in vitro* for at least six months and tested for the absence of viable *A. rhizogenes* within their tissues. After receiving authorization from the Ministry of Agriculture, Forestry and Fisheries, Japan, the plants were acclimated and grown in a greenhouse and/or field. Opine synthesis and *rolC* gene expression were analyzed to demonstrate successful introduction and expression of the transferred DNA. In total, 122 lines of variant types of dwarf plants with blue, white, and pink flowers were obtained, which might be useful for molecular breeding of a series of dwarf potted gentian cultivars.

Key words: *Agrobacterium rhizogenes*, dwarf, gentian, hairy roots, *rolC*.

The genus *Gentiana* in the family Gentianaceae includes *Gentiana triflora* and *G. scabra*, which are popular in Japan as ornamental plants and cut flowers. Several cultivars suitable for use as potted plants have also been developed in Japan, generally using growth retardants. Since these Japanese gentian cultivars comprise only limited phenotypic variation, introduction of further genetic variety (e.g. novel flower color and shape, and disease resistance) is desirable.

We previously developed a number of tools for molecular breeding, such as *in vitro* culture and a genetic transformation system (Hosokawa et al. 1996; Hosokawa et al. 2000). Although a number of transgene-introduced gentian plants have been produced by *Agrobacterium tumefaciens*-mediated transformation, the problem of transgene silencing (Mishiba et al. 2005) and delicate situation surrounding GMO development in Japan (Watanabe 2003) have hindered the production of commercial cultivars of transgenic gentian. Another approach for introducing genetic variation is *Agrobacterium rhizogenes*-mediated transformation (Tepfer 1984; Otani et al. 1993; Hoshino and Mii 1998; Yamashita et al. 2004). One of the advantages of this approach is that the transformants, derived from wild-type *A. rhizogenes*-mediated transformation (i.e. natural genetic transformation), are free from the legal controls of GMOs in Japan.

Several reports have documented successful inoculation of gentian species *with A. rhizogenes*, resulting in hairy root formation (Momčilović et al. 2001). However, among these, only a few showed regeneration from hairy roots (Suginuma and Akihama 1995; Hosokawa et al. 1997; Momčilović et al. 1997). In our previous research, we observed distinct variation, especially dwarfing, among regenerants from hairy roots of a Japanese gentian cultivar (*G. triflora*×*G. scabra*) using *A. rhizogenes* A4 strain, although the efficiencies of hairy root induction and regeneration were not high (Hosokawa et al. 1997). This result indicated that the technique could be applicable to the introduction of useful variation in breeding programs of potted gentian, provided that its utility is improved. Accordingly, we developed a more practical approach based on this previous research by: 1) using three vegetative cultivars each with different flower colors to successfully produce dwarf plants without using growth retardants, 2)

Abbreviations: MS medium, Murashige and Skoog medium (Murashige and Skoog 1962); NAA, 1-naphthaleneacetic acid; TDZ, N-phenyl-N′-(1,2,3-thiadiazol-5-yl)

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assessing the phenotypic features of a representative dwarf line over two years, 3) performing an *Agrobacterium* survival test to avoid restrictions under the plant protection law generally applied to plants obtained through infection with foreign *A. rhizogenes* strains.

Materials and methods

Hairy root induction and regeneration

In vitro plants of the interspecific hybrid (*G. triflora*×*G. scabra*) cultivar 'Polano Blue' (PB) with blue flowers and selected line 'TP1' (*G. scabra*) with pink flowers were obtained from Iwate Agricultural Research Center. Axillary buds were cultured on MS medium (Murashige and Skoog 1962) containing 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum (Wako, Osaka, Japan) in plastic culture pods (60 mm in diameter and 90 mm in height) at 20°C under cool white fluorescent lamps (35 μmol m^{-2} s^{-1}, 16 h photoperiod). Subculture was performed at 3-month intervals by transferring terminal and lateral cuttings (20–30 mm in length; 3 pieces per culture pod) to new media of the same composition, and the leafing plants were used for *Agrobacterium* infection.

The inoculation procedures followed were modified from Hosokawa et al. (1997) as follows. Wild-type *A. rhizogenes* A4 (ATCC 43057) strain harboring an agropine-type Ri plasmid (pRiA4), which was grown on YEB medium containing 15 g l^{-1} bacto-agar (Difco, USA) for 72 hours at 28°C , was inoculated directly on the stem surface and leaf tissues of the *in vitro* plants through cuts made with a scalpel. Direct-cutting inoculation was performed about 100 times per plant with caution, avoiding contact between the scalpel edge and plant medium. Co-cultivation culture was continued for 4 to 6 weeks under the same conditions as for plant culture but with a much lower light intensity (10 μmol m^{-2} s^{-1}, 16 h photoperiod).

Hairy roots induced at the inoculation sites were then excised together with segments of the original stem or leaf tissues and subcultured on MS medium containing 30 g l^{-1} sucrose, 250 mg l^{-1} cefotaxime (Claforan; Aventis Pharma, France) and 10 mg l^{-1} meropenem (Meropen; Sumitomo Pharmaceuticals, Japan; Ogawa and Mii 2000), and 2 g l^{-1} gellan gum at 20°C in darkness. For regeneration, root segments (approx. 10 mm) were transferred to MS medium containing 30 g l^{-1} sucrose, 10 mg l^{-1} N-phenyl-N′-(1,2,3-thiadiazol-5-yl) urea (TDZ), 0.1 mg l^{-1} 1-naphthaleneacetic acid (NAA), the same composition of antibiotics as that used for the root culture and 3 g l^{-1} gellan gum in plastic culture pods and cultured under the same conditions as for plant culture. The regeneration culture was subcultured every 3 weeks, and the regenerated shoots were transferred to MS medium containing 30 g l^{-1} sucrose and 3 g l^{-1} gellan gum without any antibiotics in plastic culture pods.

Agrobacterium survival test

Since the *A. rhizogenes* A4 strain used in this study was not a domestic strain, all associated experiments have to be conducted within approved facilities meeting biosafety standards under the Japanese Plant Protection Law. To avoid this restriction on the plants obtained through *A. rhizogenes* infection, an *Agrobacterium* survival test was performed with each plant according to the following protocol.

After more than 6 months of culture including at least three subcultures on antibiotic-free medium, two independent leaves were excised per each plant specimen. Leaves were crushed in 400 μl sterilized-distilled water (SDW) using a plastic pestle in a 1.5 ml microtube. Forty-μl of homogenates were batched off and mixed with 360 μl SDW in a new microtube to obtain 10-times dilution. Total volumes of 4 homogenates (2 undiluted and 2 diluted) from each plant were spread onto separate YEB plates and incubated for 5 days at 28°C in the dark. After 5 days incubation, the plates were checked for formation of bacterial colonies. The obtained colonies were identified by microscopic observation and/or by PCR analysis through comparison with colonies of the *A. rhizogenes* A4 strain. This *Agrobacterium* survival test was performed under the supervision of an inspector from the Plant Protection Station, The Ministry of Agriculture, Forestry and Fisheries, Japan. After receiving authorization, the plants were then acclimated as described by Hosokawa et al. (1997) and grown in a greenhouse or field.

Phenotypic analysis of a dwarf gentian

A dwarf line (line no. 9) obtained in a previous study (Hosokawa et al. 1997) from the white-flower cultivar 'Polano White' (PW) through inoculation with *A. rhizogenes* A4 strain was cultivated in a greenhouse and field. Four wild-type PW and 5 dwarf plants from the greenhouse, and 4 wild-type and 7 dwarf plants from the field were examined for plant height on September 3, 2001 and September 21, 2002. For examination of pollen fertility, mature pollen grains taken from 8 wild-type and 8 dwarf plants (4 greenhouse and 4 field grown plants each) were stained with aceto-carmine. Corolla length was observed at the same time.

Molecular analysis of dwarf gentians

Opine (agropine and mannopine) synthesis in hairy root tissues was confirmed by the high-voltage paper electrophoresis method described by Petit et al. (1983). For Northern blot analysis, total RNA was isolated from leaf and/or corolla tissues using Concert Plant RNA reagent (Invitrogen, Carlsbad, CA, USA) following the

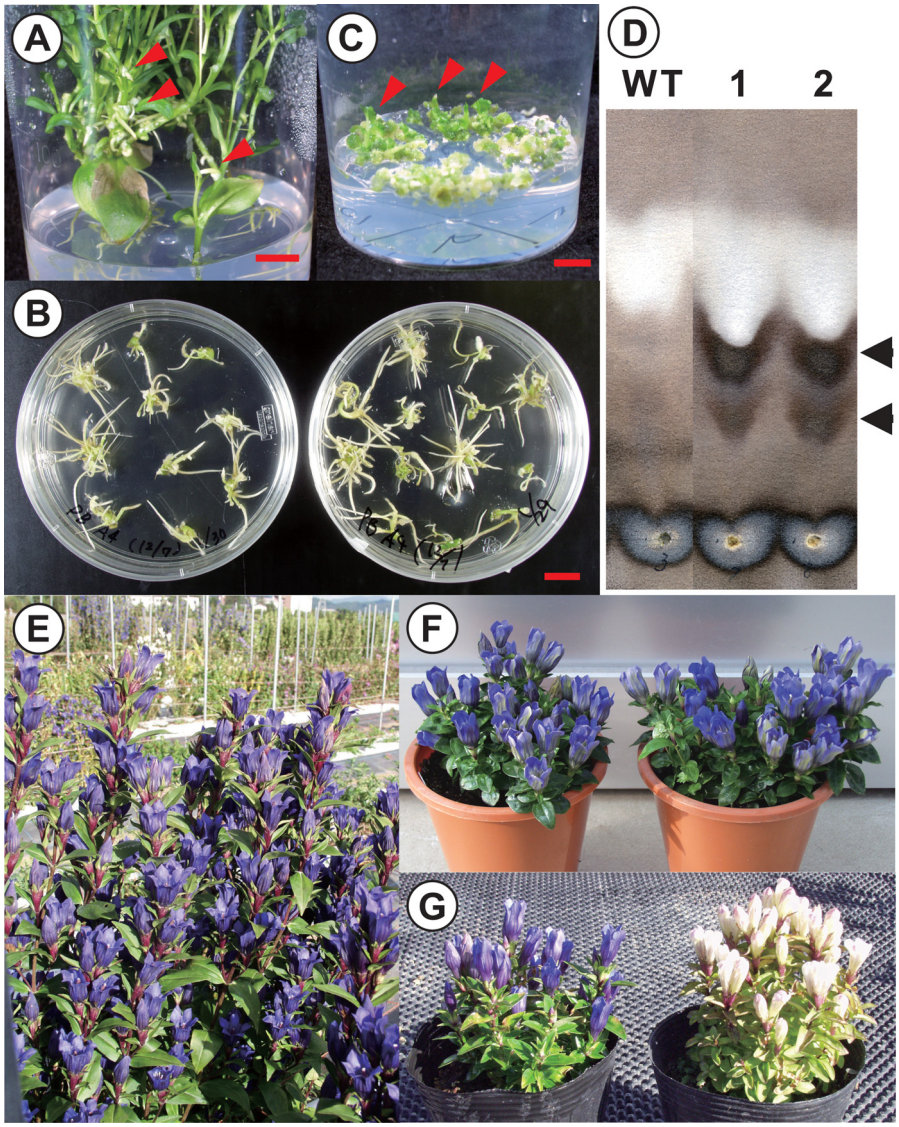

Figure 1. Production of dwarf gentian. A, Adventitious root formation from wounded parts of stem (arrowhead) inoculated with *Agrobacterium rhizogenes* A4 strain 4 weeks after inoculation. B, Growth of hairy roots on hormone-free MS medium. C, Adventitious shoot formation (arrowhead) from calli derived from hairy roots on regeneration medium. Bar=10 mm. D, Results of the agropine and mannopine assay. Extracts from root tissues of a wild-type PB plant (lane WT) and dwarf plants derived from hairy roots of PB (lanes 1 and 2) were subjected to high-voltage electrophoresis. Silver nitrate substances are indicated on the right (upper and lower arrowheads represent agropine and mannopine, respectively). Flowering of (E) wild-type PB, (F) dwarf PB, and (G) dwarf PB (left) and PW (right) plants.

supplier's instructions. Northern blot analysis of *rolC* gene expression was performed as described in our previous study (Mishiba et al. 2005). The primer pair used as the DIG-labeled probe of the *rolC* gene was 5′-ATGGCTGAAGACGACCTGTGTT-3′ (forward) and 5′-TTAGCCGATTGCAAACTTGCAC-3′ (reverse), which produces a 543-bp fragment corresponding to the *rolC* gene ORF.

Results and discussion

Hairy root formation

In our preliminary analysis of several domestic *A. rhizogenes* strains (MAFF 03-01724, 03-01725, 03-01726, 03-01727, 07-20001, 07-20002) for their ability to induce hairy roots in gentian, only the foreign A4 strain (ATCC 43057) was able to do so effectively. This strain was therefore used in this study. Generally, agropine type *Agrobacterium* strains have high-virulent pathotypes, which might be the reason for the use of these strains in most gentian studies, for hairy root induction (Momčilovič et al. 2001).

After 4 to 6 weeks of *Agrobacterium* inoculation, hairy roots emerged at many of the wound sites on both leaf and stem tissues (Figure 1A). One advantage of the hairy root induction protocol developed in the present study was the higher efficiency compared to that of our previous report (Hosokawa et al. 1997). This direct-

inoculation protocol enabled us to obtain ~100 hairy roots per plant in contrast to ~5 hairy roots per plant with our previous method. With leaf-disk inoculation, hairy roots were obtained from only 4 out of 160 leaf explants. The root segments grew successfully only when the roots were excised and cultured together with the basal tissues (Figure 1B).

Regeneration and opine analysis

In total, approx. 200 (from PB) and 150 (from TP1) hairy root lines were obtained from each independent wound and subcultured in regeneration medium. The plastic pods used for regeneration were much more suitable than plastic Petri dishes (approx. 45 and 20% hairy roots were regenerated, respectively; Figure 1C). The optimum concentration of NAA for regeneration of PB and TP1 was 0.1 mg l^{-1}, which was lower than that used for PW (1 mg l^{-1}; Hosokawa et al. 1997).

Adventitious shoot regeneration was observed in 90 (PB) and 65 (TP1) hairy root culture lines. These shoots were radicated by transferring to hormone-free medium and subcultured at least three times on medium without any antibiotics.

Root tissue extracts of two *in vitro* plants regenerated from hairy roots of PB and a wild-type PB plant were used for opine analysis. Blots corresponding to agropine or mannopine were detected on lanes of both samples of hairy root regenerant, whereas the lane of the wild-type plant showed no trace of these opines (Figure 1D).

Agrobacterium survival test

During more than three repeats of antibiotic-free subculture of the hairy root-derived plants, no proliferation of *A. rhizogenes* was observed in the plant culture medium. This was possibly due to the bacterial elimination culture using cefotaxime and meropenem or endogenous antibacterial activity of the gentian itself.

In total, 63 (PB), 44 (TP1), and 15 (PW; Hosokawa et al. 1997) lines of hairy root-derived plants were tested for the survival of *Agrobacterium* in their leaf tissues. No *A. rhizogenes* A4 strain colonies were observed, although several bacterial colonies defined as different bacteria by PCR and high resolution microscopy analysis were noted. These non-*Agrobacterium* bacteria probably resulted through contamination or endogenous bacteria in the gentian. Consequently, no *A. rhizogenes* A4 strain survived in the plant tissues after the repetitive subculture described in Materials and Methods. These results were confirmed in the presence of an inspector from the Plant Protection Station. Several months later, all plants tested received authorization from The Ministry of Agriculture, Forestry and Fisheries, Japan, for use under nonlimiting conditions.

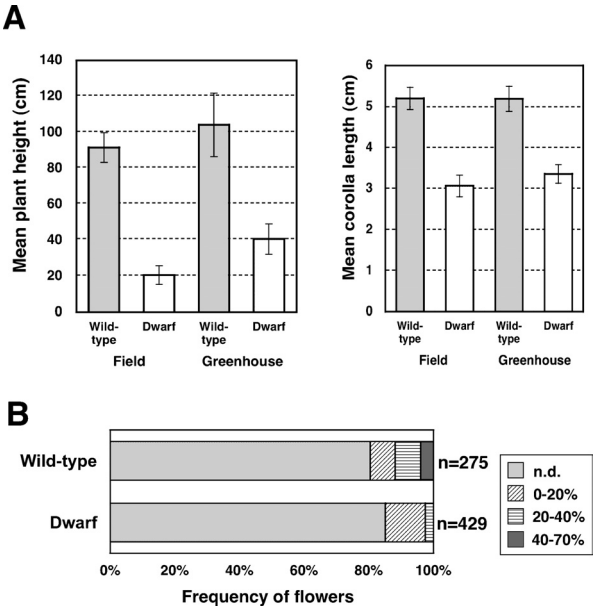

Figure 2. Phenotypic traits of the dwarf gentian. A, The mean plant height (left) and corolla length (right) of wild-type PW plants and dwarf plants (line no. 9) derived from hairy roots of PW. Plants were cultured in pots in a field or greenhouse. Data were obtained on 21 Sep 2002. Vertical bars represent standard deviations. B, Frequencies of flowers with different pollen fertilities in the dwarf (line no. 9) and wild-type PW plants. n.d. indicates no pollen grains detected in anthers.

Phenotypic features of dwarf gentians

Most of the authorized hairy root-derived plants were successfully acclimated in pots then grown in a greenhouse or field. Various morphological changes were observed in these plants: various extents of branching stems, changes in flower and leaf shapes and internodal length, and reductions in apical dominance (Figures 1E and F). To evaluate the growth characteristics of hairy root-derived plants, a representative dwarf plant line (PW line no. 9) was compared to the wild-type. Through two years evaluation, a distinct reduction in plant height was observed in the dwarf line compared with the wild-type plants (Figure 2A). Although the average plant height ratios (dwarf/wild-type) were stable in the greenhouse (40.1% and 39.1% in 2001 and 2002, respectively), those of the field plants differed between the two years (36.0% and 22.5%, respectively). The corolla length of the dwarf line was also shortened (approx. 60%) compared to the wild-type and this trend seemed to be stable in both environments (Figure 2A). Evaluation of the characteristics of the field-grown pot plants of other lines (all PB, TP1, and other PW derived-lines) are now in progress for practical use in breeding. One aim of selection is to establish a series of flowers different in color but with an identical pod shape (see Figure 1G).

Because the pollen fertility of wild-type PW was essentially low and variable among flowers, we graded pollen fertility by aceto-carmine staining into four grades and counted the numbers of flowers falling under each

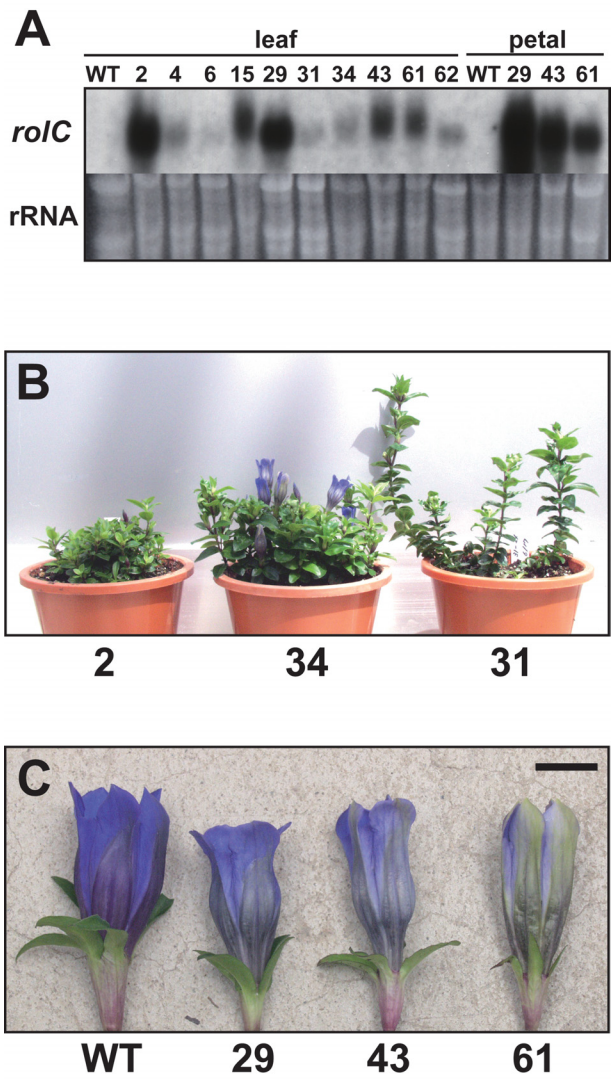

Figure 3. Expression of the *rolC* gene and flower shape. A, Results of Northern blot analysis of dwarf plant lines (line nos. 2-61) derived from hairy roots of PB and wild-type PB (WT) as a control. Ten μg of total RNA (isolated from leaf or petal tissues) was loaded in each sample. The blots were probed with a DIG-labeled *rolC* probe. B, Plant shapes of different dwarf plant lines (nos. 2, 34, 31). C, Flowers from (right to left) wild-type PB, and dwarf plant line nos. 29, 43 and 61.

for comparison between dwarf and wild-type plants. Consequently, a reduction in the proportion of flowers with higher (>20%) pollen fertilities was observed in the dwarf gentians (Figure 2B). Since a reduction in pollen (and seed) fertility in hairy root-derived plants has also been reported in other plant species (Ooms et al. 1985; Sevón et al. 1997; Koike 2003), it is perhaps a common feature (i.e. Ri-syndrome) regardless of host-plant species. Accordingly, no seeds have yet been obtained from the transformed plants by self- or cross-pollination. In general, fertility reductions allow flowers to be kept for a long time, so it is perhaps a valid character in producing a vegetative gentian cultivar.

rolC expression in the newly derived dwarf gentian

All hairy root-derived plants tested exhibited *rolC* gene expression, unlike the wild-type (Figure 3A). This result suggests that T-DNA was successfully introduced into the genomes of almost all regenerants. Various degrees of *rolC* expression were shown in relation to the extent of the dwarf phenotype (Figures 3A and B). This indicates that the *rolC* gene might play a key role in the phenotypic variation described above, which is consistent with previous reports (Nilsson et al. 1993; Winefield et al. 1999). The corolla tissues also exhibited *rolC* expression, and their extent of expression was negatively correlated with corolla length (Figures 3A and C). As shown in our recent research, the *rolC* gene promoter does not suffer transgene silencing, which strictly occurred in 35S promoter-introduced transgenic gentians (Mishiba et al. 2005). Therefore, the *rolC* promoter is a candidate transgene promoter for constitutive expression in gentian.

Perspectives

We demonstrated that genetic transformation by wild-type *A. rhizogenes* is a promising approach in formation of dwarf gentian plants. Since there are a number of plant species that can produce hairy roots with *A. rhizogenes* inoculation, this strategy will enable introduction of dwarf phenotypes in other ornamental plant species utilized for pod cultivars. Furthermore, the obtained plants are easily distinguishable from other morphologically similar cultivars, if DNA markers are developed from the boundary genomic sequence of the T-DNA integration site, enabling protection of breeders' rights scientifically. Moreover, the *A. rhizogenes*-mediated transformation established in this study was very efficient compared with the poor transformation efficiency of *A. tumefaciens* (Mishiba et al. 2005), and therefore, will probably be applicable in introduction of interesting foreign genes in gentians. In conclusion, the wild-type *A. rhizogenes*-mediated gentian transformation system presented here could be used to produce a number of phenotypic variations and is therefore a useful tool in molecular breeding.

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