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Agrobacterium tumefaciens-mediated Transformation of *Delphinium* spp.

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Abstract

A successful transformation method was reported on *Delphinium* spp., using *Agrobacterium tumefaciens*. Elongated hypocotyl and cotyledon petiole segments were infected with *Agrobacterium tumefaciens* strain LBA4404, which harbored a binary vector plasmid, pIG121-Hm, which included the β-glucuronidase (GUS) gene (with an intron) as a reporter gene, and the neomycin phosphotransferase II gene and the hygromycin phosphotransferase gene as selection markers. Explants were cultured on Murashige and Skoog medium supplemented with 1.0 mg l^{-1} thidiazuron, 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 300 mg l^{-1} ticarcillin, and 5 mg l^{-1} hygromycin or 100 mg l^{-1} kanamycin (selection medium) for regeneration. Transformation was confirmed by histochemical assays of GUS activity in plant tissues, and by PCR analysis of the GUS gene. Through four experiments, six independent GUS-positive regenerants were obtained out of 1276 explants.

Key words: *Agrobacterium tumefaciens*, *Delphinium* spp., ornamental plants, regeneration, transformation

Introduction

Genetic transformation can be instrumental in creation of novel plant cultivars. Extensive attempts have been made to improve ornamental plants by genetic engineering (Hutchinson *et al.*, 1992; Robinson and Firoozabady, 1993; Zuker *et al.*, 1998). Transformation of ornamental plants has been important, especially for the modification of ornamental characteristics such as flower longevity, color, and shape for improving marketing quality and consequently the value.

The genus *Delphinium* (Ranunculaceae) contains more than 250 species, which occur in Europe, Asia, North America, and Africa (Huxley *et al.*, 1992). With their long spikes and variety of colors, delphiniums are popular ornamental plants and are often used for cut flowers.

Although tissue culture of delphinium has been reported (Amagai and Oguri, 1994; Hosokawa *et al.*, 1998, 2001; Ohki and Sawaki, 1999), transformation of delphinium has not been published until now. A transformation system would be useful for breeding; for example, to extend flower longevity [because delphinium sepals rapidly abscise upon exposure to ethylene (Woltering and van Doorn, 1988)], modify flower color (various colors exist, including white, blue, purple, and salmon, but not brilliant red), or improve disease resistance.

In this paper, we describe an *Agrobacterium tumefaciens*-mediated transformation system for delphinium. This is the first report of the transformation of delphinium.

Materials and Methods

Plant materials for transformation

Seeds of *Delphinium* cv. 'Magic Fountains Dark Blue' were soaked in 70% ethyl alcohol for 30 s, surface-sterilized with 1% sodium hypochlorite for 1 min, and then rinsed three times with sterilized distilled water for 15 min each time. They were germinated on MS medium with 1/10-strength minerals (Murashige and Skoog, 1962), solidified with 0.8% (w/v) agar. Tissue culture of delphinium has been performed at 20 ℃ (Amagai and Oguri, 1994; Hosokawa *et al.*, 1998), so we maintained cultures at 20 ℃ throughout the experiments. Seed cultures were maintained in the dark at 20 ℃ for 19

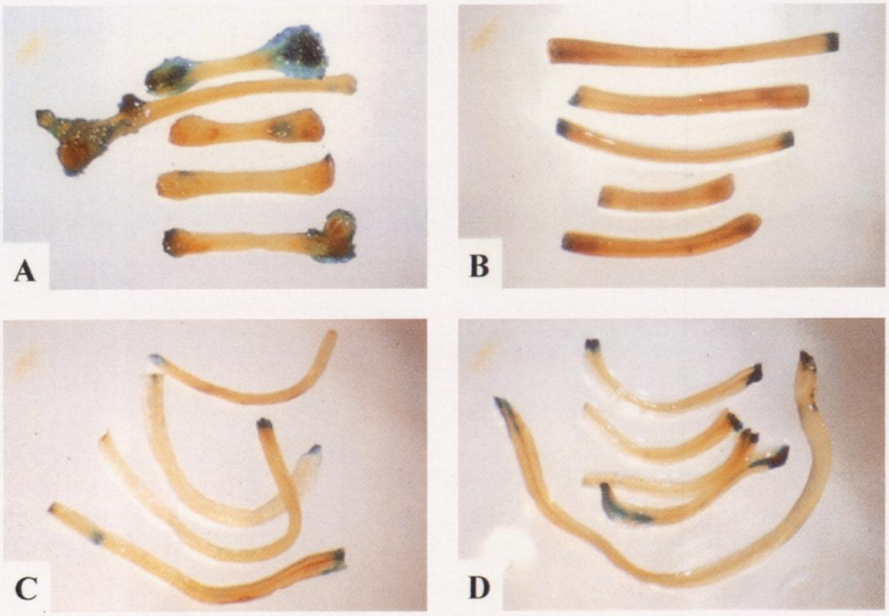

Fig. 2 Transient GUS activity after *Agobacterium* infection. After coculture with *Agrobacterium* strain LBA4404 harboring a binary vector plasmid pIG121-Hm that contains the GUS gene with an intron, all of the elongated hypocotyls with (A) or without (B) preculture and cotyledon petioles with (C) or without (D) preculture showed blue precipitation, indicating GUS activity.

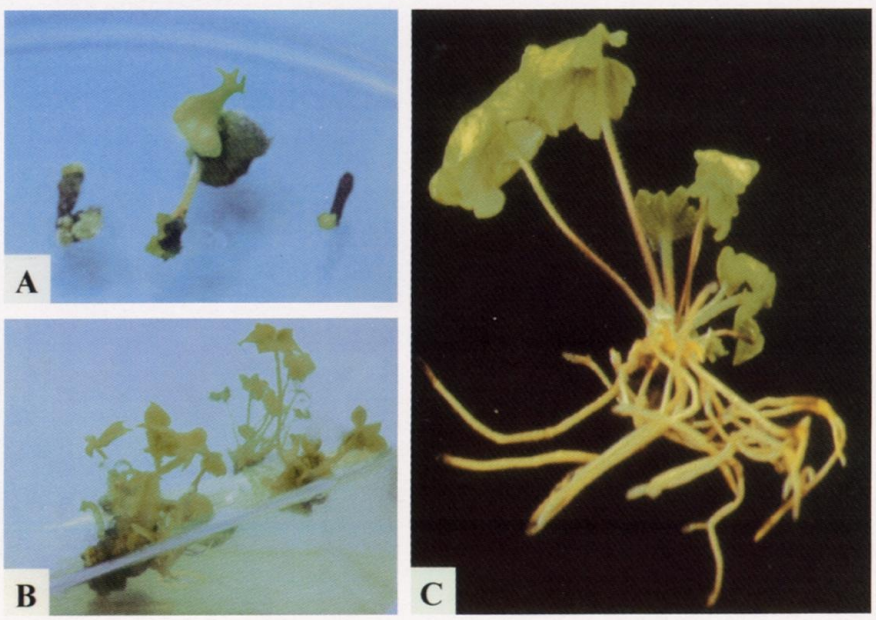

Fig. 3 Regeneration of putative transgenic delphinium. (A) Regenerated bud from explants on solid MS medium (0.2% gellan gum) containing 1.0mg l^{-1} TDZ, 1.0mg l^{-1} 2,4-D, 300mg l^{-1} ticarcillin, and 5mg l^{-1} hygromycin (selection medium). (B) Growing plants on solid MS medium (0.2% gellan gum) containing 0.1mg l^{-1} BA, 2mg l^{-1} GA_3, and 300mg l^{-1} ticarcillin (growing medium). (C) Normal delphinium plants growing on the MS-Metro Mix medium containing 300mg l^{-1} ticarcillin (6 months after *Agobacterium* infection).

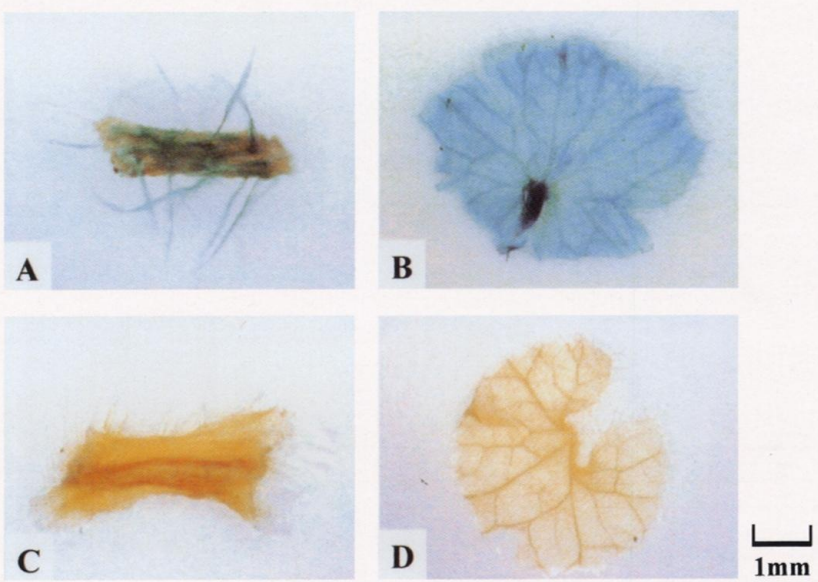

Fig. 4 GUS activity in the regeneratied plants. A segment of a petiole (A) and a leaf (B) of a regenerated plant showed blue precipitation, which indicates GUS activity. Non-transformed control plants never showed blue precipitation [(C), a segment of a petiole, (D), a leaf].

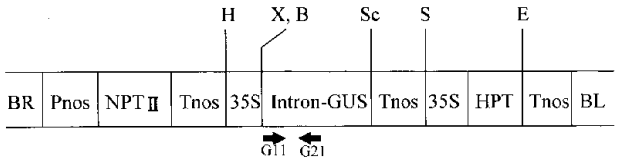

Fig. 1 Structure of the binary vector pIG121-Hm (Hiei *et al.*, 1994). The chimeric genes were inserted between the right and left border sequences of T-DNA. The GUS primer used for PCR analysis was indicated as arrows. BR and BL = right and left border sequences of T-DNA; Pnos and Tnos = promoter and terminator of nopaline synthase gene; 35S = promoter of CaMV 35S RNA gene; NPTII = coding region of neomycin phosphotransferase II gene; Intron-GUS = coding region of β-glucuronidase gene with an intron; HPT = coding region of hygromycin phosphotransferase gene; H, X, B, Sc, and S = restriction sites of *Hin*dIII, *Xba*I, *Bam*HI, *Sac*I, and *Sal*I, respectively.

days, then under cool-white fluorescent light (70 μmol m^{-2} s^{-1}, 16-h photoperiod) at the same temperature for four days without subculture. Elongated hypocotyls and cotyledon petioles were then cut into segments of about 5 mm and used as explants for transformation experiments.

Bacterial strain and vector plasmids

Agrobacterium tumefaciens strain LBA4404 (Clontech, Palo Alto, CA, USA), which harbors the binary vector plasmid pIG121-Hm (**Fig. 1**) (Hiei *et al.*, 1994), was used. pIG121-Hm contains the neomycin phosphotransferase II (NPTII) gene (nos promoter), the β-glucuronidase (GUS) gene with a modified intron from the castor bean catalase gene (Ohta *et al.*, 1990) (35S promoter), and the hygromycin phosphotransferase (HPT) gene (35S promoter).

Plasmid-bearing *Agrobacterium* cells were inoculated into liquid YEB medium (sucrose 5 g l^{-1}, beef extract 1 g l^{-1}, yeast extract 1 g l^{-1}, peptone 1 g l^{-1}) containing 50 mg l^{-1} kanamycin, 20 mg l^{-1} hygromycin, and 200 mg l^{-1} streptomycin. The culture was shaken for 24 h at 28 ℃. The cells were pelleted by centrifugation and resuspended in 10 mM magnesium sulfate solution to a density of 1.0×10^8 cells ml^{-1} for plant infection.

Preculture and coculture

Precultures and cocultures of the *Delphinium* explants were maintained in the dark at 20 ℃. Segments of elongated hypocotyls were precultured for seven days on MS medium solidified with 0.2% (w/v) gellan gum and containing 1.0 mg l^{-1} thidiazuron (TDZ), 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and 100 μM acetosyringon. Hosokawa *et al.* (2001) reported that the combination of 1.0 mg l^{-1} TDZ and 1.0 mg l^{-1} 2,4-D was one of the optimal combinations of plant growth regulators for plant regeneration of delphinium. Acetosyringon activates the virulence genes of *Agrobacterium* and enhances the transfer of foreign genes into a plant genome (Stachel *et al.*, 1985). After preculture, the explants were incubated in the *Agrobacterium* suspension for 5 min, then blotted dry on sterilized filter paper. The explants were then cocultured on a sterilized filter paper on the same MS medium for six days. Explants from cotyledon petioles were cocultured in the same manner but without preculture.

Selection and growing culture

After cocultivation, the explants were transferred to solid MS medium (0.2% gellan gum) containing 1.0 mg l^{-1} TDZ, 1.0 mg l^{-1} 2,4-D, 300 mg l^{-1} ticarcillin, and 5 mg l^{-1} hygromycin or 100 mg l^{-1} kanamycin (selection medium) for regeneration. Plates were maintained at 20 ℃ under cool-white fluorescent light (70 μmol m^{-2} s^{-1}, 16-h photoperiod). The selection medium was changed every two weeks.

About 1 month after infection, explants that formed shoots were transferred to solid MS medium (0.2% gellan gum) containing 0.1 mg l^{-1} BA, 2 mg l^{-1} GA_3, and 300 mg l^{-1} ticarcillin (growing medium), and maintained (16-h light, 20 ℃) to allow regenerated plants to grow. Addition of GA_3 is known to stimulate elongation of adventitious shoots (Aida and Shibata, 1996). After two months' culture on the growing medium, a single regenerated plant was excised from each explant and cultured on sterilized Metro-Mix 350 (bark ash product; Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) containing MS solution with half-strength minerals and 300 mg l^{-1} ticarcillin (Metro-Mix medium; Aida *et al.*, 1999) for rooting and further growth (16-h light, 20 ℃).

GUS assay and PCR analysis

A petiole segment and a leaf disk were excised from each regenerated plant for histochemical GUS assay. Histochemical GUS activity was examined by the procedure reported by Jefferson *et al.* (1987) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GLUC) as a substrate. The GUS assay buffer used in this experiment contained 20% methyl alcohol to eliminate endogenous GUS activity (Kosugi *et al.*, 1990). The samples were incu-

bated at 37 ℃ for 16 h.

PCR template DNA was obtained from leaf tissues of the regenerants according to the method described by Edwards *et al.* (1991). A set of primers that amplify a 970-bps fragment were constructed from the GUS DNA sequence (Aida and Shibata, 1995). The primer sequences were as follows: G11, 5'-CCCTTATGTTACGTCCTGTAGAAACCC-3'; G21, 5'-CCAATCCAGTCCATTAATGCGTGGTCG-3'. Takara Ex-*Taq* DNA polymerase was used for PCR amplification, and 25 μl of reaction mixture was prepared according to the manufacturer's directions (Takara Shuzo, Kyoto, Japan). The PCR amplification conditions were as follows: 2 min of preheating at 94 ℃ ; 35 cycles of 30 s at 94 ℃ , 2 min at 60 ℃ , and 1 min at 72 ℃ ; and a final post extension of 9 min at 72 ℃ . The amplification product was separated on 1% agarose gel and stained with ethidium bromide.

Results

Transient GUS assay after Agrobacterium infection

In a preliminary examination, we assessed the ability of etiolated hypocotyls and cotyledon petioles to produce gene-transferred explants, by detection of the transient GUS activity that represents early infection by *Agrobacterium* strain LBA4404. Five explants with or without seven days preculture were assayed from each tissue. All examined explants showed at least one blue precipitation at the cut surface, which indicates GUS activity. Precultures for seven days seemed to enhance gene transfer for etiolated hypocotyls (**Fig. 2A, B**), however, there are no significant difference for cotyledon petioles (**Fig. 2C, D**). Vector pIG121-Hm contains a modified GUS gene that can be expressed only in plant cells (Ohta *et al.*, 1990). This result shows that the GUS gene was transferred to the delphinium cells and was successfully expressed.

Regeneration

One to two months after *Agrobacterium* infection, adventitious buds regenerated from calli appeared at the cut surface of explants (**Fig. 3A**). Because excised buds tended to die, the explants with adventitious buds were transferred to the growing medium without cutting. After transfer to the growing medium, the buds became green and grew gradually (**Fig. 3B**). By 6 months after infection, we obtained 131 independent plants from 1276 explants through four experiments (**Table 1**). Regenerated plants grew normally (**Fig. 3C**) after the transplant to the MS-Metro Mix medium.

GUS assay

We examined the 131 regenerated plants for GUS activity. The assay showed that six plants were GUS-positive and that non-transformed control plants never showed GUS activity (**Fig. 4**), suggesting that the GUS-positive plants were transgenic. It seemed that both elongated hypocotyls and cotyledon petioles could produce transformants (**Table 1**).

PCR analysis

We selected six GUS-positive regenerated plants (**Table 1**) for PCR analysis. The GUS-positive plants had a single band at 970 bp, the same position as the band from pIG121-Hm. Wild plants had no band (**Fig. 5**).

Table 1. Transformation efficiency in *Delphinnium* with binary vector pIG121-Hm

Experiment	Explants	Antibiotics for selection (mg l^{-1})	No. of Explants	No. of Regenerants[1)]	Regeneration rate (%)	No. of GUS-positive plants[2)]	Efficiency(%) (transformants per explant)
1	Elongated hypocotyls	Kanamycin (100)	255	13	5.1	0	0
2	Elongated hypocotyls	Hygromycin (5)	331	57	17.2	3	0.9
3	Cotyledon petioles	Kanamycin (100)	240	11	4.6	1	0.5
4	Cotyledon petioles	Hygromycin (5)	450	50	11.1	2	0.4

[1)]Only a single regenerant was collected from each explant to obtain independent transformants.

[2)]Transformation was confirmed by histochemical GUS assay.

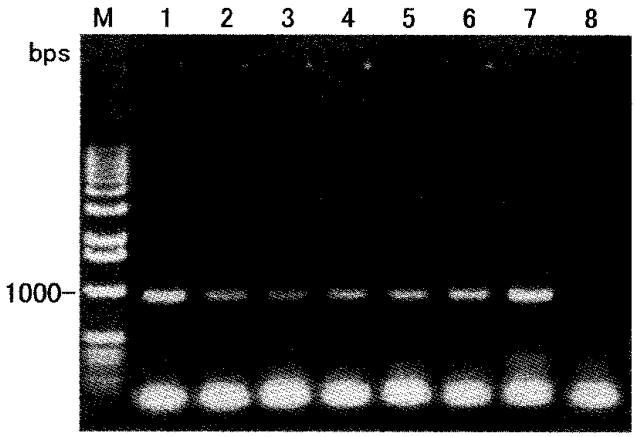

Fig. 5 PCR analysis of GUS-positive (GUS+) plants. PCR products amplified from upstream regions in GUS-ORF with primer pairs G11 and G21. Lanes 1 to 3, GUS-positive regenerant from elongated hypocotyls selected with hygromycin; lane 4, GUS-positive regenerant from cotyledon petioles selected with kanamycin; lanes 5, 6, GUS-positive regenerant from cotyledon petioles selected with hygromycin; lane 7, pIG121-Hm binary vector DNA (positive control); lane 8, wild-type plant (negative control); lane M, 1-kb ladder. The GUS-positive plants and the positive control all showed 1 band at 970 bp. The negative control showed none.

Discussion

In early studies of the tissue culture of delphinium, shoots were used as explant material, but contamination caused by parasitic microorganisms was a serious problem (Bott, 1980). The use of tissues of seedlings grown from surface-sterilized seeds can overcome this problem. We used this method, using etiolated hypocotyls and cotyledon petioles as explants for the transformation experiments. Seed culture was maintained in the dark to obtain etiolated hypocotyls, since normal hypocotyls of delphinium were too short to use as explants.

We based the characterization of gene transfer on GUS gene expression. We used vector pIG121-Hm (Hiei *et al.*, 1994), which contains GUS with a modified intron from the castor bean catalase gene in the coding region (Ohta *et al.*, 1990). Prokaryotes cannot express the intron-GUS combination, so the GUS expression in the explants and regenerated plants was due to transgene expression in the plant cells. There was no detectable endogenous GUS activity in the delphinium tissues (**Fig. 4C, D**).

The experiment used selection medium containing TDZ and 2,4-D as plant growth regulators for bud regeneration. TDZ is highly efficient at shoot regeneration in a wide variety of plants (Huetteman and Preece, 1993), and 2,4-D is known to stimulate adventitious organogenesis from delphinium tissue (Hosokawa *et al.*, 1998). Adventitious buds appeared at the cut surface of explants 1 to 2 months after *Agrobacterium* infection (**Fig. 3A**), and regenerated plants grew normally (**Fig. 3C**). The combination of 1.0 mg l^{-1} TDZ and 1.0 mg l^{-1} 2,4-D used in this study seemed to be effective for organogenesis of adventitious buds from elongated hypocotyls and cotyledon petioles of delphinium.

The PCR analysis confirmed the existence of the GUS gene into the GUS-positive plants. Pieces of leaf blades and petioles were cut from the six GUS-positive regenerated plants and transferred to YEB medium containing 50 mg l^{-1} kanamycin, 20 mg l^{-1} hygromycin, and 200 mg l^{-1} streptomycin, where they were incubated at 28 ℃ for a week. All samples showed no bacterial contaminants (data not shown). The presence of contaminating *Agrobacterium* harboring the vector in its tissues should be detected by this test. The GUS-positive plants showed a band at 970 bps, indicating at least single-copy integration of the GUS gene into the genome. We considered the GUS-positive plants to be transformants.

Following the results of the preliminary experiment, we used 5 mg l^{-1} hygromycin or 100 mg l^{-1} kanamycin to select transformants, both of which suppressed organogenesis from non-infected segments of elongated hypocotyls and cotyledon petioles (data not shown). Both hygromaycin and kanamycin could be used for selection of transgenic delphinium (**Table 1**).

Many transgenic plants with reduced ethylene production and ethylene sensitivity have been reported (Chang *et al.*, 1993; Savin *et al.*, 1995; Aida *et al.*, 1998). Delphinium flowers are sensitive to ethylene; their sepals abscise rapidly on exposure to it. Our transformation system may be able to produce delphinium transformants with longer flower life.

There have also been many reports of the modification of flower color by genetic transformation. The cases are on: petunia (Meyer *et al.*, 1987; van der Krol *et al.*, 1988, 1990; Napoli *et al.*, 1990; Holton *et al.*, 1993; Helariutta *et al.*, 1993; Bradley *et al.*, 1995; Tanaka *et al.*, 1995; Davies *et al.*, 1998;), Arabidopsis (Lloyd *et al.*, 1992), gerbera (Elomaa *et al.*, 1993), lisianthus (Deroles *et al.*, 1995; Schwinn *et al.*, 1997), chrysanthemum (Courtney-Gutterson *et al.*, 1994), and rose (Souq *et al.*, 1996). Our transformation system could also

be useful for creating new colors of delphinium, such as brilliant red that would be valuable in the market.

It could also improve other characteristics, such as resistance to disease. The expression of a rice chitinase gene gave increased resistance to black-spot disease in rose (Marchant *et al.*, 1998) and to gray mold in chrysanthemum (Takatsu *et al.*, 1999). The introduction of a chitinase gene into delphinium might enhance its resistance to diseases such as powdery mildew, which is one of the most serious constraints in delphinium production.

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