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Original Paper

Establishment of *Agrobacterium*-mediated genetic transformation system in *Dahlia*

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Abstract An efficient system for *Agrobacterium*-mediated transformation was established in dahlia (*Dahlia*×*pinnata*) 'Yamatohime'. Mass of shoot primordia (MSP) induced on MS medium supplemented with 10 mg l^{-1} TDZ were inoculated with *Agrobacterium tumefaciens* strain EHA101 (pIG121-Hm) harboring both β-glucuronidase (GUS) and hygromycin resistant genes. After 2 days of co-cultivation, the MSP were transferred to a selection medium containing hygromycin with meropenem for bacterial elimination. Shoots were successfully regenerated from survived MSP on hormone-free medium without hygromycin and they rooted on hormone-free medium containing hygromycin. The hygromycin-resistant plants thus obtained showed histochemical blue staining for GUS. Transformation of plants was confirmed by PCR and Southern blot analyses. Transgenic 'Kokucho' were also produced by using the same transformation procedure, suggesting wide applicability of this *Agrobacterium*-mediated transformation procedure for other dahlia cultivars.

Key words: *Agrobacterium tumefaciens*, *Dahlia*×*pinnata*, ornamental plant, shoot regeneration, transformation.

Dahlia (*Dahlia*×*pinnata*) has been grown as a popular tuberous ornamental crop used for cut flower and as pot and garden plants. For cultivation, dahlias are always facing to extinction because of the high susceptibility to some viruses such as dahlia mosaic virus (Pappu et al. 2005). In addition, although dahlia has a large variation in flower color and morphology, there have been constant demands for novel type of flowers such as blue flowers. Unfortunately, no germplasms are available for conferring these useful characters in dahlia. Although it is now expected to utilize genetic transformation methods to achieve these breeding objectives, there have been no reports on the genetic transformation in dahlia.

Agrobacterium-mediated transformation method has most widely been used for various plant species due to its feasibility without any special equipments and appropriate insertion of one or a few transgene copies (Gelvin 2003). Since dahlia is known to be susceptible to infection with *A. tumefaciens*, which causes crown gall disease (Loper and Kado 1979), this method is expected to be effective for genetic transformation of this plant. For Asteraceae plants, there have been some reports of genetic transformation by using *Agrobacterium*-mediated method (Evenor et al. 2006; Frulleux et al. 1997; Godoy-Hernández et al. 2006). However, there has been no report on the successful *Agrobacterium*-mediated transformation of dahlia. In the present study, we describe an efficient protocol for *Agrobacterium*-mediated genetic transformation method using dahlia cultivar 'Yamatohime' as a target material.

Materials and methods

Plant material

MSP were induced as described elsewhere (Otani et al. submitted) from leaf explants of *in vitro* plants of dahlia cultivar 'Yamatohime' on MS medium (Murashige and Skoog 1962) containing 10 mg l^{-1} thidiazuron (TDZ) (Wako Pure Chemical Industries, Osaka, Japan) and 30 g l^{-1} sucrose and solidified with 2.5 g l^{-1} gellan gum (Gelrite; Wako Pure Chemical Industries, Osaka, Japan) and subcultured every month on the same medium. MSP of 'Kokucho', 'Amy K' and 'L'Ancresse' were also induced from leaf explants of *in vitro* plants using the same method and subcultured every month on the same medium but reducing TDZ to 1 mg l^{-1}. The pH of medium was adjusted to 5.8 before autoclaving (15 min at 121°C) and all the cultures were incubated at 25°C under a 16 h photoperiod at 35 μmol m^{-2} s^{-1} with cool white fluorescent light. The MSP about 14 days after subculture were divided into small pieces (ca. 5 mm in diameter) and used for the inoculation of *Agrobacterium*.

Plasmid vector and bacterial strain

A. tumefaciens strain EHA101 (Hood et al. 1986), which

Abbreviations: GUS, β-glucuronidase; HF, hormone-free; MSP, mass of shoot primordia; MS, Murashige and Skoog; TDZ, thidiazuron.
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harbors a binary vector pIG121-Hm (Ohta et al. 1990) that contains a hygromycin phosphotransferase gene (*hpt*) and an intron-*gus* gene both under the control of a 35S cauliflower mosaic virus promoter, and a neomycin phosphotransferase II gene (*nptII*) under the control of a nopaline synthase promoter in the T-DNA region, was used.

Sensitivity of MSP and shoots to hygromycin

To determine the optimum concentrations of hygromycin for the selection of transformed cells, MSP were placed on the MS medium containing 10 mg l^{-1} TDZ and different concentration of hygromycin (Hygromycin B; Wako Pure Chemical Industries, Osaka, Japan) (0, 10, 20, 40 mg l^{-1}). The MSP were incubated at 25°C under a 16 h photoperiod and subcultured at 2 week-intervals. The experiment was repeated 3 times, each with 50 MSP per treatment. The survival rates of MSP were determined after 2 months of culture.

Shoots were placed on the hormone-free (HF) MS medium containing different concentration of hygromycin (0, 5, 10 mg l^{-1}). The shoots were incubated at 25°C under a 16 h photoperiod and the rooting rates of shoots were determined after 1 month of culture. The experiment was repeated 3 times, each with 18 shoots per treatment.

Transformation and transgenic plant regeneration

A. tumefaciens strain was grown for 20–24 h in LB medium supplemented with 50 mg l^{-1} kanamycin (Kanamycin sulfate; Wako Pure Chemical Industries, Osaka, Japan), 50 mg l^{-1} hygromycin, and 25 mg l^{-1} chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) by agitating at 120 rpm using a reciprocal shaker to give a density of OD_{600}=1.2. For inoculation, about two gram fresh weight of MSP were transferred to 40 ml liquid inoculation medium, which was MS medium supplemented with 100 μM acetosyringone (3′,5′-dimethoxy-4′-hydroxy-acetophenone; Sigma-Aldrich, St. Louis, MO, USA), 30 g l^{-1} sucrose and 10 mg l^{-1} TDZ in a 100 ml flask. Two or 0.4 ml of *Agrobacterium* suspension culture was added to each flask and some flasks were subjected to sonication treatment for 5 min at 60% (24 kHz) ultrasound power (Transsonic Digital S, Elma, Germany). After infection with or without sonication treatment, each flask was vacuumed for 5 min. Then the MSP were blotted dry with Kimwipes (Nippon Paper Crecia Company, Tokyo, Japan), co-cultivated for 2 days on the co-cultivation medium, which was the same as the inoculation medium but solidified with 2.5 g l^{-1} gellan gum, at 20°C or 25°C in the dark. After co-cultivation, the MSP were transferred onto selection medium, which was the same as the co-cultivation medium but lacking acetosyringone and containing 10 mg l^{-1} hygromycin for the selection and 40 mg l^{-1} meropenem trihydrate (Meropen; Dainippon Sumitomo Pharma, Osaka, Japan; Ogawa and Mii 2007) for eliminating *Agrobacterium*. After culturing for one week, MSP were subcultured on the same medium. Ten days after the subculture, MSP were transferred to the same medium, in which, however, hygromycin concentration was elevated to 20 mg l^{-1}. Two weeks after the transfer, they were again transferred to the same medium but with the elevated concentration of hygromycin to 40 mg l^{-1}. MSP surviving on this medium were further proliferated on the same medium with subcultures at every two weeks. The hygromycin resistant MSP thus obtained were then transferred onto the same medium but lacking hygromycin and cultured for 1 month. They were then transferred for shoot regeneration onto HF MS regeneration medium containing 30 g l^{-1} maltose instead of 30 g l^{-1} sucrose and 20 mg l^{-1} meropenem but lacking hygromycin, which was solidified with 8 g l^{-1} agar (Wako Pure Chemical Industries, Osaka, Japan). After elongation of the regenerated shoots on the same medium, they were excised from MSP and cultured on 2.5 g l^{-1} gellan gum-solidified HF MS medium containing 30 g l^{-1} sucrose, 20 mg l^{-1} meropenem and 10 mg l^{-1} hygromycin for rooting, which was expected to occur only on transgenic shoots.

GUS assay

Three months after culturing on selection medium, hygromycin resistant MSP were subjected to stable histochemical GUS assay (Jefferson et al. 1987; Stomp 1992). Leaves of putative transgenic plants were also subjected to the GUS assay.

Tissue samples were incubated in sodium phosphate buffer containing 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) as the substrate for 15 h at 37°C after vacuum-infiltration with the buffer solution by using desiccator for 10 min. The stained tissue samples were then soaked in 70% ethanol to remove the chlorophyll.

Polymerase chain reaction analysis and Southern hybridization

Genomic DNA was isolated from leaves (0.3 g) of the putative transgenic plants and an untransformed control plant following CTAB method (Murray and Thompson 1980), in which the time of incubation at 65°C after adding 2% CTAB solution was modified from 30 min to 6 h to remove stickiness of the extracted DNA samples.

Genomic DNA was used for PCR analysis to detect the presence of *hpt* and *gus* genes in the putative transgenic plants. PCR amplifications were performed in 20 μl reaction mixture containing 0.5 U TaKaRa Ex Taq polymerase and 1×Ex Taq buffer (Takara Shuzo, Shiga, Japan), 0.2 mM each dNTP, 0.5 mM each primer and 50 ng of template DNA. The PCR was performed according to the following thermal cycles: 30 cycles of 94°C for 1 min (denaturation), 59°C (62°C for *gus*) for 1 min (annealing) and 72°C for 1.5 min (elongation), using the following set of primers, 5′-ACA GCG TCT CCG ACC TGA TGC A-3′ and 5′-AGT CAA TGA CCG CTG TTA TGC G-3′ for *hpt* (Xiao and Ha 1997), 5′-GGT GGG AAA GCG CGT TAC AAG -3′ and 5′- GTT TAC GCG TTG CTT CCG CCA -3′ for *gus* (Hamill et al. 1991). After amplification, 4 μl of PCR products were loaded on the gel and detected by ethidium bromide staining after electrophoresis on 0.9% agarose gel at 100 V for 30 min.

For Southern hybridization, 15 μg of genomic DNA was digested with *Hin*dIII, which cut single site within T-DNA, and electrophoresed on a 0.8% agarose gel. The DNA was then transferred to nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co., Billerica, MA, USA). The *gus* probe (1.2-kb) was generated from pIG121-Hm by labeling with digoxigenin (DIG) using the PCR DIG Probe Synthesis kit (Roche Diagnostics, Mannheim, Germany) and the following set of primers, 5′-GGT GGG AAA GCG CGT TAC AAG -3′ and 5′-GTT TAC GCG TTG CTT CCG CCA -3′ (Hamill et al. 1991), were used. Pre-hybridization and hybridization were carried out using high-SDS hybridization buffer containing 50% deionized formamide, 5×SSC, 50 mM sodium phosphate (pH 7.0), 2% blocking solution, 0.1% *N*-lauroylsarcosine and 7% SDS. Washing and detection were performed according to the instruction manual of the DIG labeling and Detection System (Roche Diagnostics, Mannheim, Germany). For detection of hybridization signals, membrane was exposed to a detection film (Lumi-Film Chemiluminescent Detection Film; Roche Diagnostics, Mannheim, Germany) for 2 h.

Data Analysis

Data obtained for the GUS positive MSP formation were subjected to the analysis of variance (ANOVA) using the SPSS statistical package. Tukey's HSD test was performed to identify significant differences among the treatments, with significance level of $p<0.05$.

Results and discussion

Sensitivity of MSP and shoots to hygromycin

Efficient selection is a necessary prerequisite for successful production of transgenic plants through *Agrobacterium*-mediated method. Our results indicate that MSP growth and shoot rooting was extremely sensitive to hygromycin. When MSP were cultured on medium containing 40 mg l^{-1} hygromycin for 5 weeks, all the MSP turned brown and died (Figure 1A). On the other hand, rooting of shoots was inhibited by lower concentration of hygromycin. When shoots were cultured on the 5 mg l^{-1} hygromycin containing medium for 1 month, all the shoots stopped growing and failed to root (Figure 1B).

Effect of infection condition on transformation

After co-cultivation for 2 days with *A. tumefaciens* EHA101 (pIG121-Hm), MSP were transferred onto selection medium, on which they turned brown gradually. However, green MSP of putative transformants initiated to appear from a part of some inoculated MSP one and a half months after transferring onto selection medium. Three months after the transfer, these hygromycin resistant MSP reached approximately 5–10 mm in diameter (Figure 2A) and all of them showed histochemical GUS staining, which was however, not

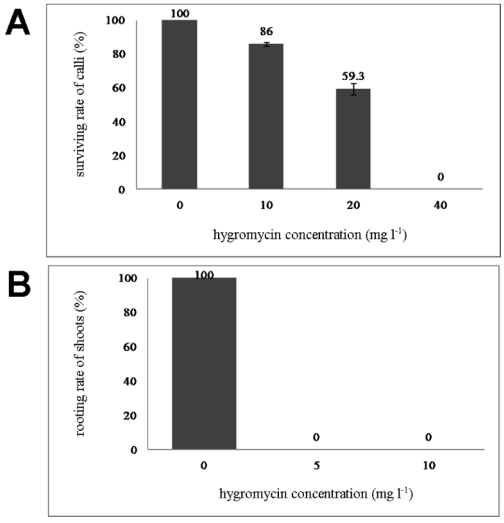

Figure 1. Hygromycin sensitivity to dahlia cultivar 'Yamatohime'. (A) Surviving rate of MSP and (B) Rooting rate of shoots on hygromycin-containing medium.

observed in untransformed control MSP (Figure 2B). To produce transformants effectively, the influence of some factors of inoculation and co-cultivation of *Agrobacterium*, i.e., dilution of the bacterial suspension, sonication treatment and co-cultivation temperature were examined (Table 1). Higher concentration of bacteria, i.e., less dilution of *Agrobacterium* liquid culture ($OD_{600}=1.2$) with 40 ml of liquid MS medium (1 : 20 ratio), generally gave more GUS positive MSP (1.3–2.6 times as high) than that diluted at 1 : 100 ratio, except in the case where sonication was not applied and co-cultivated at 25°C. In *Agrobacterium*-mediated transformation, sonication has been used to enhance transformation in various species (Amoah et al. 2001; Shrestha et al. 2007; Tang 2003; Trick and Finer 1997, 1998; Weber et al. 2003; Zaragoza et al. 2004). Similarly, in the case of dahlia, sonication increased transformation efficiency in comparison with the untreated control (1.4–2.6 times as high), except for the case where dilution of bacterial suspension was at 1 : 100 ratio and co-cultivated at 25°C. The temperature during the co-cultivation period for 2 days in the dark also affected the transformation efficiency evaluated by GUS stainability. In all the conditions where other two treatments were the same, 25°C gave 1.4–5.2 times higher transformation efficiency than 20°C. Among the eight conditions tested, combination of 1 : 20 dilution of the bacterial suspension, application of sonication treatment, and co-cultivation at 25°C was the best although no statistically significant differences were found among all the treatments (Table 1).

Regeneration of plantlets from GUS positive MSP

After continuous subculture at 2 week-intervals on medium containing 10 mg l^{-1} TDZ, 40 mg l^{-1}

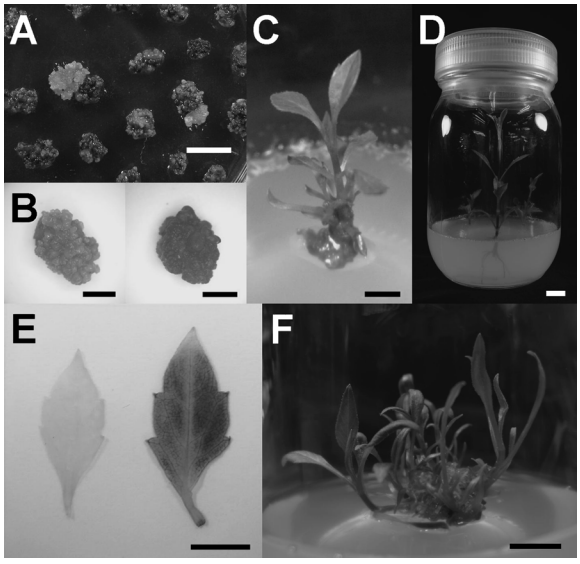

Figure 2. Production of transgenic plants of dahlia. (A) The emergence of hygromycin resistant MSP of 'Yamatohime' three months after culture on the selective medium containing 10 mg l^{-1} TDZ. bar=10 mm (B) Stable GUS expression on MSP of transgenic (right) and non-transformed (left) 'Yamatohime'. bar=10 mm (C) Regenerated shoots from GUS positive MSP of 'Yamatohime' on hygromycin-lacking HF medium. bar=5 mm (D) Rooting of regenerated shoot of 'Yamatohime' 1 month after the transfer onto hygromycin-containing HF MS medium. bar=10 mm (E) Stable GUS expression on leaf of transgenic 'Yamatohime' plant (right) and non-transformed plant (left). bar=10 mm (F) Regenerated shoots from GUS positive MSP of 'Kokucho' on hygromycin-lacking HF medium. bar=10 mm

Table 1. Effect of inoculation condition on the transformation of MSP.

Dilution of bacterial suspension	Sonication (5 min, 24 kHz)	Co-cultivation temperature (°C)	No. of GUS positive MSP/(g)*
1:20	−	20	1.77±0.39
	−	25	2.95±1.36
	+	20	3.08±1.31
	+	25	4.20±0.57
1:100	−	20	0.69±0.34
	−	25	3.61±0.23
	+	20	1.79±0.48
	+	25	3.20±1.00

Three repeats were performed and the data were evaluated 3 months after initiation of the culture on selection medium. *Each value represents a mean±SE of the three independent experiments. No statistically significant differences at $p<0.05$ were found among the 8 treatments by Tukey's HSD test.

meropenem and 40 mg l^{-1} hygromycin for 1–2 months, the hygromycin-resistant and GUS positive MSP selected were transferred onto medium containing 10 mg l^{-1} TDZ and 20 mg l^{-1} meropenem but lacking hygromycin. After culturing for 1 month on this medium without subculture, the MSP were transferred onto HF regeneration medium lacking hygromycin. The MSP successfully regenerated into shoots 2–3 months after transferring onto regeneration medium (Figure 2C). Regenerated and elongated shoots of 3–4 cm long were excised from MSP, and then transferred onto HF MS medium containing 10 mg l^{-1} hygromycin for rooting and selecting transformed shoots. Root formation occurred from all the shoots 1 to 2 weeks after the transfer (Figure 2D). Leaves of hygromycin resistant plantlets thus obtained were subjected to the GUS assay. A whole extent of the leaf showed blue staining, which was not observed in untransformed leaf (Figure 2E). Regenerated plantlets with roots were transferred to pots containing fine-grained soil and acclimatized for 2 to 3 weeks in a growth chamber at 22°C under a 14 h photoperiod. Totally five plants were then transferred to the greenhouse. Most of them exhibited a normal phenotype and produced normal single flowers but one of them produced double flowers 4 months after the transfer.

In case of 'Kokucho', transformation was performed on the best conditions established for 'Yamatohime', i.e., combination of 1:20 dilution of the bacterial suspension, application of sonication treatment, and co-cultivation at 25°C. Hygromycin resistant MSP were successfully obtained and they showed blue staining by GUS assay. As a result, 34 GUS positive MSP were obtained from 3 g (about 200) MSP inoculated. This transformation efficiency of 'Kokucho' (No. of GUS positive MSP/inoculated MSP (g)=11.3) was higher than that of 'Yamatohime' (No. of GUS positive MSP/inoculated MSP (g)=4.2). The GUS positive MSP were regenerated into shoots after transfer onto HF regeneration medium (Figure 2F). Leaves of the regenerated shoots also showed blue staining in whole by GUS assay, which was not observed in non-transformed leaf.

Molecular analysis

PCR analysis showed that all of the eleven selected putative transformants had positive bands of the *hpt* (0.6 kb) and *gus* (1.2 kb) genes (Figure 3A), indicating that the present transformation procedure achieved strict selection of transformants. Southern hybridization using the *gus* gene as the probe also showed the hybridization signals in all the eleven selected lines with the insertion of one to three copies of the gene (Figure 3B, lanes 1–11), whereas no signal was detected in the untransformed plant (Figure 3B, lane N).

In this study, we have successfully established an *Agrobacterium tumefaciens*-mediated genetic transformation procedure leading to production of transgenic plants in dahlia for the first time, using MSP of two cultivars 'Yamatohime' and 'Kokucho' as materials for *Agrobacterium* inoculation. We have also successfully produced transgenic plants in other two cultivars, 'Amy K' and 'L'Ancresse' by using the same genetic transformation methods (data not shown). Therefore, it is highly possible that these methods can be used for transformation of many other dahlia cultivars. Further

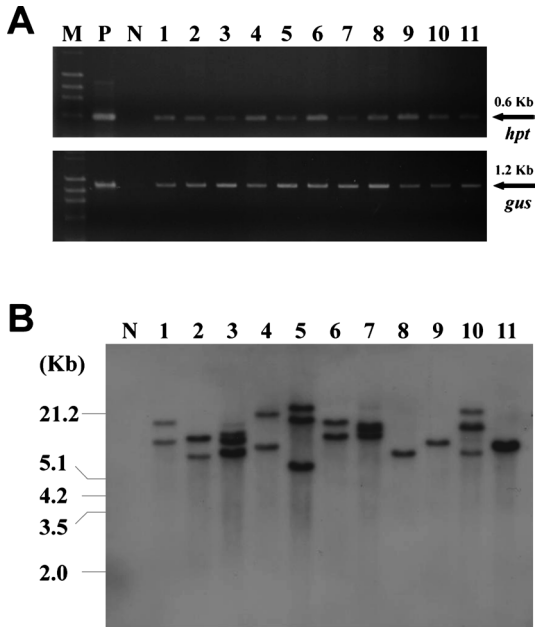

Figure 3. Molecular analysis of transgenic plants. (A) PCR analysis of transgenic dahlia plantlets for the hygromycin phosphotransferase (*hpt*) and β-glucuronidase (*gus*) genes. Lane *M* DNA size markers (λ/*Hin*dIII, ϕX174/*Hae*III); *P* plasmid pIG121-Hm (positive control); *N* non-transformed plant as control; 1–11 transgenic plants of dahlia. Arrows: Positions of the expected 0.6- and 1.2-kb fragments for *hpt* and *gus* genes, respectively. (B) Southern blot analysis of transgenic plants. Genomic DNA was digested with *Hin*dIII and hybridized with digoxigenin (DIG)-labelled *gus* gene probe. Lanes: *N* non-transformed plant, 1–11 transgenic plant.

studies are now in progress for producing transgenic plants of dahlia with commercially important traits such as virus resistance and flower color by using this method.

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