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Note

High efficiency *Agrobacterium*-mediated transformation of *Dendrobium* orchid using protocorms as a target material

Wasana Phlaetita, Dong Poh Chin, Ntui Valentine Otang[a], Ikuo Nakamura, Masahiro Mii*[,b]

Graduate School of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan
*E-mail: miim@faculty.chiba-u.jp Tel & Fax: +81-4-7137-8177

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Abstract Dendrobium orchid is one of the most popular cut flower and potted plants. In this study, a protocol for efficient genetic transformation of *D. nobile*-type orchids was established by co-cultivating 21 day-old protocorms for 3 days with *Agrobacterium tumefaciens* strain EHA101 carrying pIG121Hm harboring *β*-glucuronidase (*GUS*) gene as reporter gene and hygromycin phosphotransferase (*hpt*) gene as selectable marker gene. After selection of the infected protocorms on New Dogashima (ND) medium containing 10 g l^{-1} maltose, 30 mg l^{-1} hygromycin and 20 mg l^{-1} meropenem for 3 months followed by the culture on hygromycin-free recovery medium for 1 month, secondary protocorm-like bodies (PLBs) produced on this medium were again transferred onto secondary selection (regeneration) medium. Plantlets were successfully regenerated from these secondary PLBs after the transfer. The highest transformation efficiency of 27.3% was obtained when protocomrs were inoculated with 10 times diluted *Agrobacterium* solution (OD_{600}=0.1) for 300 min. Transformation of the selected plants was confirmed by GUS assay, PCR and Southern blot analysis. This protocol could be adopted to produce transgenic *D. nobile*-type orchids with various traits such as novel flower color and resistances to biotic and abiotic stresses.

Key words: *Agrobacterium tumefaciens*, *Dendrobium nobile*-type hybrid, genetic transformation, orchid, protocorm.

Dendrobium is one of the largest genera in the family Orchidaceae and includes the most popular orchids grown for commercial production of cut flowers and pot plants. The commonly cultivated species include *D. phalaenopsis*, *D. gouldii* and *D. nobile*. Recently, cultivars with novel flower colors, shapes and disease resistance have been developed through intraspecific and interspecific hybridizations (Kamemoto et al. 1999). However, conventional breeding in this genus by means of sexual hybridization is restricted due to long generation time, generally 3 years from seed sowing to flowering and lack of usable genetic variability (Kuehnle and Sugii 1992). Therefore, genetic engineering remains an important tool for producing *Dendrobium* with desirable traits such as blue flowers, disease resistance and long flower life.

In the last 15 years, genetic transformation of orchids has been reported using *Agrobacterium*-mediated method by targeting protocorm-like bodies (PLBs) as material for *Phalaenopsis* (Belarmino and Mii 2000; Chai et al. 2002; Sjahril and Mii 2006), *Cymbidium* (Chen et al. 2002; Chin et al. 2007), *Vanda* (Shrestha et al. 2007), *Cattleya* (Zhang et al. 2010), *Oncidium* (Liau et al. 2003) and *Dendrobium* (Men et al. 2003b; Nan et al. 1998; Yu et al. 2001). It is natural to use PLBs as the target for genetic transformation because most cultivars of these commercially important orchids are vegetatively propagated by tissue culture, and hence targeting the cultivars with superior traits is usually needed. In transgenic plants, however, expression of foreign genes is sometimes affected greatly by the genetic background of the target plants (Cogan et al. 2001). Therefore, it is necessary to introduce such genes into the plants with diverse genotypes to evaluate the usefulness or effectiveness of the target genes for transformation. Based on such idea, Mishiba et al. (2005) have already reported successful *Agrobacterium*-mediated transformation with rapid selection of transgenic plants in *Phalaenopsis* by targeting protocorms at an early stage after germination. In *D. nobile*, however, protocorms have not been used as target material although PLBs have successfully been transformed by using both biolistic and *Agrobacterium*-mediated methods (Men et al. 2003a, 2003b)

In the present study, we tried to develop a simple

Abbreviations: AS, acetosyringone; CTAB, cetyltrimethylammonium bromide; *gus*, *β*-glucuronidase; *hpt*, hygromycin phosphotransferase; ND, New Dogashima; *nptII*, neomycin phosphotranferase.

[a] Present address: King Abdullah University of Science and Technology, Thuwal, Saudi Arabia
[b] Present address: Center for Environment, Health and Field Sciences, Chiba University, Kashiwanoha 6-2-1 Kashiwa, Chiba 277-0882, Japan

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and efficient procedure for *Agrobacterium*-mediated transformation by using the protocorms obtained from the cross between two cultivars of *D. nobile*-type hybrid as target material.

Seeds of *Dendrobium nobile*-type hybrid obtained from a cross between *D.* Flower Palace 'Cinderella' × *D.* Santa Isabel 'True Love' were kindly provided by Mr. Nobuyuki Asai, Asai Daikeien Co., Ltd. To produce protocorms, seeds were surface-sterilized by agitating for 10 min in a sodium hypochlorite solution containing 1% (v/v) active chlorine, and then rinsed three times with sterile distilled water. Sterilized seeds were cultured in 35 ml of liquid New Dogashima (ND) medium (Tokuhara and Mii 1993) containing 10 g l^{-1} maltose without adding any plant growth regulators (pH 5.4) in 100 ml flask and agitated at 80 rpm. All of the seeds were incubated at 25°C under constant illumination (33 μmol m^{-2} s^{-1}) with cool white fluorescent lamps (National FL30SN, Osaka, Japan).

Agrobacterium tumefaciens strain EHA101 (Hood et al. 1986), harboring binary vector pIG121Hm (Ohta et al. 1990), which contains a hygromycin phosphotrasferase 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.

Agrobacterium was grown overnight at 28°C in LB liquid medium containing 50 mg l^{-1} hygromycin (Hygromycin B; Wako Pure Chemical Industries, Ltd., Osaka, Japan), 50 mg l^{-1} kanamycin (Kanamycin sulfate; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 25 mg l^{-1} chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA). The bacterial suspension was diluted to final density of OD_{600}≈1.0 in inoculation medium, which was liquid ND medium containing 100 μM acetosyringone (AS) (3′,5′-dimethoxy-4′-hydroxy-acetophenone; Sigma-Aldrich, St. Louis, MO, USA). For inoculation, 21 day-old protocorms (Mishiba et al. 2005) obtained as described above were put into 35 ml inoculation medium in a 100 ml flask, which was then added with 3.5 ml (1/10) or 700 μl (1/50) of *Agrobacterium* suspension culture to give 1/10 or 1/50 dilution, and incubated for either 30 or 300 min with mild agitation at 80 rpm on rotary shaker. The protocorms were then collected with a 40 μm nylon mesh placed on a funnel, immediately transferred with the nylon mesh onto a 0.25% (w/v) gellan gum (Wako Pure Chemical Industries, Ltd.)-solidified ND medium supplemented with 100 μM AS, and co-cultivated for 3 days.

For selection and elimination of bacteria, protocorms were washed with liquid ND medium and placed onto 40 μm nylon mesh, which was overlaid on 0.25% (w/v) gellan gum-solidified ND medium containing 30 mg l^{-1} hygromycin and 20 mg l^{-1} meropenem trihydrate (Meropen; Dainippon Sumitomo Pharma, Osaka, Japan http://www.ds-pharma.co.jp), a β-lactam antibiotic (Ogawa and Mii 2004, 2007), for primary selection of transformants. The protocorms were subcultured every 3 weeks to fresh medium of the same composition. After selection for 3 months, protocorms showing green coloration were cut transversely into two pieces and placed onto ND medium without hygromycin but with meropenem (recovery medium) (Mishiba et al. 2005) and cultured for 1 month. Then, newly proliferated PLBs from protocorms on this medium were selected and returned to selection medium containing 30 mg l^{-1} hygromycin and 10 mg l^{-1} meropenem for 1 month (secondary selection). Transformation efficiency was recorded after 1 month of secondary selection as the percentage of initial protocorms yieldling survived PLBs from 3 replicated experiments. Data collected were subjected to statistical analysis after arcsine transformation and were compared using one way ANOVA, followed by Tukey's multiple range test using SPSS program (IBM SPSS statistical version 22).

After secondary selection, PLBs were transferred onto ND medium containing 10 mg l^{-1} hygromycin and 10 mg l^{-1} meropenem for shoot formation and rooting. Some putative transgenic plantlets were transferred to pots and grown in growth chamber at 25°C under a 16-/8-h (day/night) photoperiod without any acclimatization treatment.

After 5 months of selection, hygromycin-resistant plantlets were randomly selected and subjected to histochemical GUS assay (Jefferson 1987) with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) as the substrate. Leaves and roots of control and transformed plantlets were incubated in sodium phosphate buffer containing X-Gluc overnight at 37°C after vacuum-infiltration with the buffer solution using desiccator for 15 min. Following the incubation, tissues were bleached with 70% ethanol until chlorophyll was removed.

Total DNA was extracted from leaves (1.5 g fresh weight) of the control and putative transgenic plants following CTAB method (Murray and Thompson 1980). PCR detection of *hpt* and *gus* was performed as described by Hamill et al. (1991) and Xiao and Ha (1997), respectively. The PCR amplifications were carried out using the following thermal cycles: 30 cycles of 94°C for 1 min (denaturation), 59°C for *hpt* and 62°C for *gus* for 1 min (annealing) and 72°C for 1.5 min (elongation). Primers used for amplifying a 0.6-kb fragment inside the *hpt* gene were 5′-ACA GCG TCT CCG ACC TGA TGC A-3′ and 5′AGT CAA TGA CCG CTG TTA TGC G-3′ and those amplifying a 1.2 kb fragment of the *gus* gene were 5′-GGT GGG AAA GCG CGT TAC AAG-3′ and 5′-GTT TAC GCG TTG CTT CCG CCA-3′. After amplification, 3 μl of PCR products were loaded on the gel and detected by ethidium bromide staining after

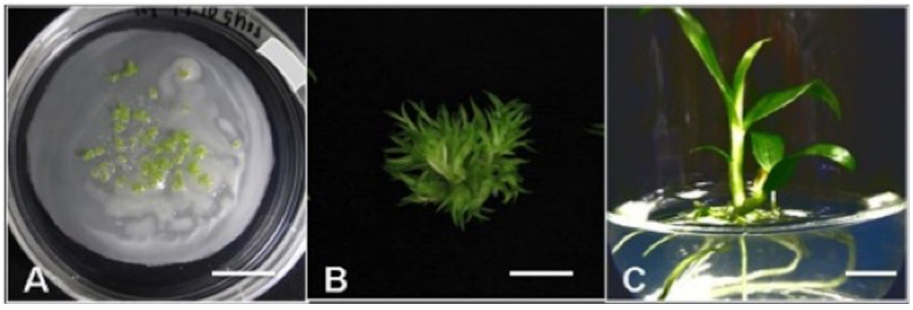

Figure 1. Regeneration of putative transgenic plantlets of *Dendrobium nobile*-type hybrid. A: Inoculated protocorms with *A. tumefaciens* (EHA101/pIG121Hm) showing bacterial overgrowth after 3 days of co-cultivation. B: Multiple shoots regenerated from protocorm-derived PLBs after secondary selection with 30 mg l^{-1} hygromycin and 10 mg l^{-1} meropenem for 4 months. C: Putative transgenic plantlet 8 months after transfer one of the multiple shoots and culture on medium containing 10 mg l^{-1} hygromycin and 10 mg l^{-1} meropenem. *Bars*=1 cm.

Table 1. Effect of bacterial concentration and inoculation time on transformation efficiency of protocorms in *Dendrobium nobile*-type hybrid.

Inoculation time (min)	Bacterial concentration	Total no. of protocorm	Total no. of Hm-resistant protocorms	Regenerated Hm-resistant protocorms (%)
30	1/10	510	71	13.8±0.5^{c}
	1/50	379	52	13.4±0.5^{c}
300	1/10	515	139	27.3±0.5^{a}
	1/50	453	103	22.8±1.2^{b}

Transformation efficiency was recorded after secondary selection on medium containing 30 mg l^{-1} hygromycin and 10 mg l^{-1} meropenem as the number of independent Hm resistant protocorms with respect to the initial number of protocorms infected. Means of 3 replications with the different letters show significant difference as analyzed by Tukey's test at $p<0.05$.

electrophoresis on 1% agarose gel at 100 V for 35 min. For Southern hybridization, 10 μg of genomic DNA was digested overnight with *Hin*dIII, electrophoresed on 0.9% agarose gel, and subsequently transferred to a nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co, Billerica, MA, USA). The GUS probe (1.2 kb) was generated from plasmid DNA of pIG121Hm 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′ were used. Washing and detection were carried out according to the instruction manual of the DIG labeling and detection system (Roche Diagnostics, Mannheim, Germany). For detection of hybridization signals, the membrane was exposed to a detection film (Lumi-film Chemiluminescent Detection Film; Roche Diagnostics, Mannheim, Germany) for 60 min.

Our preliminary study showed that prolonged co-cultivation period of 4 days caused rapid browning of protocorms in *D. nobile*-type hybrid after transfer onto selection medium, whereas no appreciable damage was observed in the co-cultivation of up to 3 days. After 3 days of co-cultivation, used in the present study, bacterial overgrowth was observed around protocorms infected for 300 min at 1 : 10 bacterial concentration (Figure 1A), whereas protocorms infected for 30 min did not show bacteria overgrowth irrespective of the bacterial concentration. In both cases, some of the infected protocorms continued to grow and retained their green color after transfer onto selection medium containing antibiotics, while all of the control protocorms without *Agrobacterium* infection failed to grow and turned brown within 4 weeks of culture on selection medium. Although bacterial overgrowth during the selection stages is usually considered as a serious problem in *Agrobacterium*-mediated genetic transformation (Arifin et al. 2004), it was successfully prevented by using 20 mg l^{-1} meropenem in the present study even if the overgrowth was observed during the co-cultivation period.

Previously, Mishiba et al. (2005) succeeded to obtain transgenic plants in *Phalaenopsis* by infecting 21 day-old protocorms with the same *Agrobacterium* strain, EHA101 (pIG121Hm). Although we did not examine the optimum stage of protocorm development for *Agrobacterium* infection, present results clearly indicate the appropriateness of the selected developmental stage of protocorms as the target material in *D. nobile*-type hybrid.

In the present study, inoculation time of *Agrobacterium* solution gave apparently high effect on transformation efficiency and long inoculation time of 300 min resulted in higher transformation efficiencies (23–27%) than short inoculation time of 30 min, which gave 13–14% efficiencies (Table 1). On the contrary, Men et al. (2003b) obtained the highest transformation efficiency by infecting PLBs of *D. nobile* with AGL1/pCAMBIA1301 vector with the short inoculation time (30 min) and indicated that the long period of 60 min

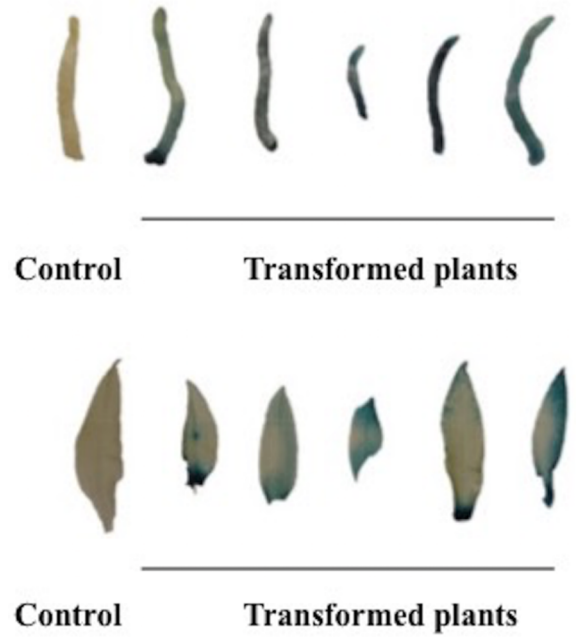

Figure 2. GUS histochemical assay of roots (upper) and leaves (lower) of transgenic *Dendrobium nobile*-type hybrid.

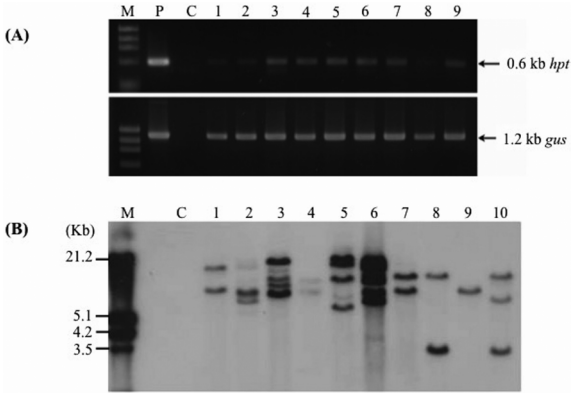

Figure 3. Molecular analysis of transgenic *Dendrobium nobile*-type hybrid. (A) PCR analysis of transgenic plantlets for hygromycin phosphotransferase (*hpt*) and *β*-glucuronidase (*gus*) genes. Lane M: Molecular size marker (*ϕ*X174/*Hae*III), Lane P: Plasmid pIG121Hm (Positive control), Lane C: untransformed plant as negative control (non-transformed plant), Lanes 1-9: Transgenic plants. (B) Southern blot analysis of transgenic plants. Ten micrograms of genomic DNA was digested with *Hin*dIII and hybridized with digoxigenin (DIG)-label *gus* gene probe. Lane M: Molecular size marker (DIG), Lane C: untransformed plant as negative control (non-transformed plant), Lanes 1–10: Transgenic plants.

significantly decreased transformation efficiency to 3–4%. In *Phalaenopsis*, bacterial inoculation period of 7 h was used for production of transgenic plants when protocorms were used as a target (Mishiba et al. 2005), whereas 2 h was found to be optimal for the transformation of PLBs (Sjahrill and Mii 2006). These results suggest that the long period of inoculation is suitable for transformation of orchids when using protocorms as target materials. Although the reason for the difference in the optimum time of infection with *Agrobacterium* for transformation efficiency between protocorm and PLB, it might be attributed to the differences in the strain of *Agrobacterium* or in the susceptibility between these two types of target materials.

Although usefulness of high bacterium concentration has been reported as an imported factor for achieving transformation of orchids (Chin et al. 2007; Mishiba et al. 2005; Shrestha et al. 2007; Zhang et al. 2010), it has less effect in the present study, i.e. 1 : 10 dilution (OD_{600}≈0.1) gave slightly higher transformation efficiency than 1 : 50 dilution only at 300 min inoculation time and no differences was found at 30 min inoculation (Table 1).

The surviving protocorms obtained from the primary selection were cut into 2 pieces and cultured on recovery medium lacking hygromycin. One month after the culture on this medium, PLBs were regenerated from the surviving tissues of protocorms. Some of PLBs survived after transfer onto the same selection medium used for the secondary selection of the transgenic tissues and regenerated into shoots (Figure 1B). They produced roots after transfer to medium containing reduced concentration of hygromycin to 10 mg l^{-1} and the same concentration (10 mg l^{-1}) of meropenem (Figure 1C).

The leaf and root tissues of plantlets regenerated on the secondary selection medium showed positive results for histochemical GUS assay (Figure 2). About 90% of the selected plants showed GUS staining in these tissues (data not shown), whereas GUS activity was not detected in any of the tissues of untransformed plantlets. To confirm the presence of transgenes in the putative transformants, PCR analysis was carried out for the *hpt* and *gus* genes. All selected hygromycin-resistant plants showed positive amplification for both genes (Figure 3A), which were not detected in the untransformed plants, indicating that the T-DNA of the binary vector was successfully integrated into the genome of the transgenic plants. Southern hybridization was also performed on randomly selected plantlets using *GUS* probe. All the plants showed hybridization signals with the integration of one to five copies of T-DNA in the plant genome (Figure 3B, lanes 1–10), while no hybridization signal was observed in the untransformed plant (Figure 3B, Lane C).

In conclusion, we have established a highly efficient transformation system with 27.3% transformation efficiency in *D. nobile*-type hybrid using protocorms as target material by applying 300 min period of inoculation of *Agrobacterium* solution at OD_{600}≈0.1. The value is much higher than the highest transformation efficiencies of *Dendrobium* obtained previously by 18% with *Agrobacterium*-mediated (Men et al. 2003b) and 19.87% with microprojectile bombardment methods (Suwanaketchanatit et al. 2007), respectively. Therefore, the transformation procedure established in the

present study could be used efficiently to introduce various desirable traits such as novel flower colors and disease resistances into *D. nobile*-type hybrids with various genetic background at the same time. Although the reason for the high transformation efficiency of protocorms in *D. nobile*-type hybrid is still unclear, protocorms might also be useful material to obtain comparably high transformation efficiency in other orchid species of *Dendrobium* and other economically important orchid genera. For producing *D. nobile*-type hybrids with blue flower, introduction of flavonoid 3′,5′-hydroxylase gene is now in progress by using the transformation system established in this study.

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