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Original Paper

Agrobacterium-mediated transformation of protocorm-like bodies in *Dendrobium* Formidible 'Ugusu'

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Abstract *Agrobacterium*-mediated genetic transformation system was established in *Dendrobium* Formidible 'Ugusu' by inoculating PLBs with *A. tumefaciens* strain EHA101 (pIG121Hm) harboring hygromycin phosphotranferase (*hpt*) and neomycin phosphotranferase II (*nptII*) genes as selectable marker gene and *β*-glucuronidase (*gus*) gene as a reporter gene. For obtaining the optimum conditions for the transformation, several factors such as the stage of PLBs after subculture, bacterial concentrations, kind of inoculation medium, inoculation time and inoculation condition (with or without rotary shaking), were examined. After inoculation, PLBs were cocultivated for 3 days and then transferred for selection onto 2.5 g l^{-1} gellan gum-solidified ND medium containing 10 g l^{-1} maltose, 20 mg l^{-1} hygromycin and 20 mg l^{-1} meropenem. Hygromycin-resistant plantlets were regenerated from secondary PLBs after 4 months of selection. Transformation of these plants was confirmed by GUS histochemical assay, PCR and Southern blot analyses. The highest transformation efficiency of 18.5% was obtained when PLBs 3 weeks after subculture were inoculated with 1 : 10 diluted bacteria (OD_{600}≈0.1) with liquid medium containing only 10 g l^{-1} maltose and 100 μM acetosyringone with shaking for 30 min.

Key words: *Agrobacterium tumefaciens*, *Dendrobium* Formidible, Orchid, Ornamental plant, protocorm-like bodies.

Dendrobiums are commercially important orchids cultivated as potted plants and cut flowers around the world because of their beautiful flowers and good shape. Nowadays, numerous cultivars have been developed through intra- and inter-specific cross hybridizations. However, improvements are still required in various traits such as flower longevity, disease resistance and novel flower color in order to catch up with its increasing demand and extended market. For achieving these breeding goals, conventional breeding technologies are rather difficult to apply, and genetic transformation remains an useful and possible option.

In *Dendrobium* orchids, genetic transformation studies has been initiated by using biolistic-mediated method (Chia et al. 1994; Kuehnle and Sugii 1992; Tee et al. 2003; Suwanaketchanatit et al. 2007), followed by *Agrobacterium*-mediated method (Men et al. 2003). Among these studies, successful regeneration of transgenic plants has been reported by Men et al. (2003) and Suwanaketchanatit et al. (2007). Based on these studies, virus resistant plants were successfully produced by introducing viral coat proteins in the genome of *Dendrobium* (Chang et al. 2005). The inhibition of ethylene production in order to delay the senescence and increase the flower longevity in *Dendrobium* has also been reported recently (Zheng et al. 2012).

Dendrobium Formidible is a name given to the interspecific hybrid between *D. formosum* and *D. infundibulum* in 1967 (The International Orchid Register, Royal Horticultural Society, http://apps.rhs.org.uk/horticulturaldatabase/orchidregister/). Most cultivars of Formidible have large white flowers (ca. 12 cm in diameter) with yellow eye and usually bloom during early to mid summer season. Moreover, each flower lasts long for more than one month. Due to these unique characters, it has become an important popular pot plant during the hot summer season. Since *D.* Formidible lacks color variations and novel cultivars with other flower colors are now expected because of difficulty in crossing with other *Dendrobium* species and cultivars, genetic transformation technology is now expected to apply to achieve such breeding goals. In the present study, we developed a simple and efficient system for *Agrobacterium*-mediated transformation in *D.* Formidible 'Ugusu,' which is a dwarf cultivar suitable as pot plant, by using protocorm-like bodies (PLBs) as a

Abbreviations: AS, acetosyringone; BA, N^6-benzyldenine; CTAB, cetyltrimethylammonium bromide; *gus*, *β*-glucuronidase; *hpt*, hygromycin phosphotransferase; NAA, 1-naphthaleneacitic acid; ND, New Dogashima; *nptII*, neomycin phosphotranferase; PLBs, protocorm-like bodies.

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target material.

Materials and methods

Plant materials

PLBs of *Dendrobium* Formidible 'Ugusu' were induced by culturing shoot tips (1 mm high×2 mm diameter) excised from sprouting shoots of ca. 10 cm on New Dogashima (ND) medium (Tokuhara and Mii 1993) containing 10 g l^{-1} maltose, 0.1 mg l^{-1} 1-naphthaleneacitic acid (NAA), 1.0 mg l^{-1} N^6-benzyldenine (BA) and 0.25% gellan gum at pH 5.4 and were subculture every 4 weeks for 2 years. For each subculture, PLBs were cut into several pieces of approximately 3–4 mm in size and transferred to the same fresh medium but added with 10 g l^{-1} potato granules (Basic American Foods, California, USA). One month-old PLBs after each subculture were used for the transformation experiments. To investigate the effect of the age of PLBs after subculture on transformation, PLBs maintained for the different periods (0, 1, 2, 3, 4, 5 and 6 weeks) after subculture on the same medium were used for the experiment. All of the cultures were incubated at 25°C under constant illumination with cool-white-fluorescent lamps (Hitachi FLR40SW/M/36-B 25HA, Japan).

Plasmid vector and bacterial strain

Agrobacterium tumefaciens strain EHA 101 (Hood et al. 1986) harboring the binary vector plasmid pIG121Hm (Ohta et al. 1990) was used for the transformation. The T-DNA region of pIG121Hm contains hygromycin phosphotrasferase gene (*hpt*) and neomycin phosphotransferase II gene (*nptII*) as selectable markers and an intron-containing β-glucuronidase (*gus*) reporter gene.

Inoculation and cocultivation with Agrobacterium

Agrobacterium was grown overnight at 28°C in LB liquid medium containing 50 mg l^{-1} hygromycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 50 mg l^{-1} kanamycin (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, PLBs of 3–4 mm in size were transferred to 35 ml inoculation medium in a 100 ml flask, to which *Agrobacterium* suspension was added to give 1/10 or 1/50 concentration of bacteria (OD_{600}=0.1 or 0.02), and incubated for either 30, 45 or 60 min with or without rotation for shaking at 100 rpm. Effect of the shorter inoculation times, i.e., 10, 20, 30 and 40 min, on the transformation efficiency was also examined under optimal concentration of bacteria (OD_{600}=0.1) with the rotary shaking. For selection of the suitable inoculation medium, PLBs were infected with *Agrobacterium*, which was suspended in either distilled water (DW), 1% maltose solution without ND nutrient (only 1% Mal) or full strength ND medium with 1% maltose. AS was added to these 3 kind of inoculation media at 100 μM. The inoculated PLBs were then blotted dry, and co-cultivated on 2.5 g l^{-1} gellan gum (Wako Pure Chemical Industries, Ltd., Osaka, Japan)-solidified ND medium supplemented with 100 μM AS at 25°C in the dark for 3 days. After determining the optimum inoculation condition for the transformation, the suitable stage of PLBs for the transformation was investigated.

Agrobacterium *elimination, selection and plant regeneration*

After cocultivation, the inoculated PLBs were washed with liquid ND medium, blotted dry and transferred onto 2.5 g l^{-1} gellan gam-solidified ND medium containing 20 mg l^{-1} hygromycin and 20 mg l^{-1} meropenem (Ogawa and Mii 2004, 2007; Meropen; Dainippon Sumitomo Pharma, Osaka, JPN) for selection and bacterium elimination (primary selection). PLBs were subcultured every 2 weeks to a fresh medium of the same composition. Secondary PLBs showing green coloration were separated from the inoculated original PLBs by forceps after 2 months of culture on the selection medium and cultured on hygromycin-free fresh medium for 1 month. Thereafter, the PLBs were returned onto the selection medium as used for the primary selection for 2 months. For plantlet regeneration, surviving PLBs were transferred onto gellan gum-solidified ND medium containing 10 mg l^{-1} hygromycin and 10 mg l^{-1} meropenem. Regenerated plantlets as putative transformants were each transferred into a culture bottle containing the same selection medium. Three months after transplanting, plantlets which developed 3–4 leaves and several roots were acclimatized in sphagnum moss of small pots and grown in a growth chamber at 25°C under a 16-/8-h (day/night) photoperiod. Transformation efficiency was recorded after 4 month of culture on the secondary selection medium as the percentage of initial PLBs yieldling survived PLBs. The experiment was replicated 3 times and the data were subjected to statistical analysis after arcsine transformation and compared using one way ANOVA, followed by Tukey's multiple range test using SPSS program (IBM SPSS statistical version 22).

GUS assay

GUS histochemical assay was performed on hygromycin-resistant PLBs, shoots, leaves, roots and untransformed tissues as a control. These tissues were immersed in X-Gluc (5-bromo-4-chloro-3-indolyl-D-glucuronide) solution (Jefferson 1987), vacuum-infiltrated for 15 min and incubated overnight at 37°C. The tissues were then soaked in 70% ethanol for several hours to remove chlorophyll before observation.

DNA isolation and molecular analysis

Plantlets regenerated from PLBs were selected randomly to check for the presence of transgene. Total genomic DNA was extracted from leaf tissues (2.0 g fresh weight) of the plantlets using the CTAB method (Murray and Thompson 1980). For detecting *hpt*, *nptII* and *gus*, the

PCR amplifications were carried out using the following thermal cycles: 30 cycles with 94°C for 1 min (denaturation), 59°C for *hpt*, 58°C for *nptII* and 62°C for *gus* for 1 min (annealing), respectively 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′; those amplifying a 0.7-kb fragment inside the *nptII* gene were 5′GAG GCT ATT CGG CTA TGA CTG-3′ and 5′-ATC GGG AGC GGC GAT ACC GTA-3′, and those for 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′, respectively. After amplification, 3 μl of PCR products were loaded on the gel and detected by ethidium bromide staining after 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 transferred to a nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co, Billerica, MA, USA). The *hpt* probe (0.6 kb) was generated from plasmid DNA of pIG121Hm by labeling with digoxigenin (DIG) using the PCR DIG Probe Synthesis kit (Roche Diagnostics, Mannheim, Germany). Southern blot hybridization and detection were performed using digoxigenin-labeled *hpt* probe following the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Results and discussion

Effect of bacterial concentration, inoculation time and inoculation condition on the transformation efficiency

PLBs were infected with two concentrations of *A. tumefaciens* (OD_{600}=0.1 or 0.02) at different inoculation time (30, 45, 60 min) with or without rotary shaking during inoculation. As the results, higher concentration of bacteria, shorter inoculation period and application of agitation gave beneficial effects on the transformation and the highest transformation efficiency of 12.2% was obtained when 1 : 10 (OD_{600}=0.1) bacterial concentration and 30 min inoculation time was applied with shaking during inoculation (Table 1). Especially at this bacterial concentration and inoculation period, 1.8 times higher transformation efficiency was obtained with the application of agitation during the inoculation period than that without it. These results indicate that agitation treatment increased the susceptibility of explants to *Agrobacterium* through giving the damage of the surface of PLBs. For confirming the optimum period of inoculation, PLBs were further inoculated with *Agrobacterium* at the periods for 10 to 40 min at the bacterial concentration of OD_{600}=0.1 with agitation. The results showed that 30 min of inoculation time gave the highest transformation efficiency of 13.9%, whereas shorter inoculation time as 10 and 20 min decreased to 2.8 and 5.6%, respectively and also longer inoculation time as 40 min decreased the efficiency to 6.9% (Figure 1). These results indicate that 30 min was the optimum period for the infection of PLBs with *Agrobacterium* at OD_{600}=0.1 with agitation treatment during inoculation period. In other *Dendrobium* orchids, inoculation of PLBs for 30 min also gave the highest transformation efficiency (Men et al. 2003; Yu et al. 2001). However, in other orchids, combinations of higher bacterial concentration and longer inoculation period have been recommended for the successful genetic transformation, such as $OD_{600}\approx$0.5 for 4 h in *Vanda* (Shrestha et al. 2007) and $OD_{600}\approx$0.6 for 4 h in *Cattleya* (Zhang et al. 2010), respectively. Therefore, *Dendrobium* orchids might be more susceptible to *Agrobacterium* infection compared to other kind of orchids.

Table 1. Effect of bacterial concentration and inoculation time on transformation of *Dendrobium* Formidible 'Ugusu.'

Conditions for transformation system			Total No. of PLBs inoculated	No. of Hm-resistant PLBs [1)]	Transformation efficiency [2)]
Inoculation condition	Bacterial concentration	Inoculation time (min)			
Shaking	1 : 10	30	90	13	12.2±0.5 [a]
		45	90	7	6.7±0.5 [b]
		60	90	6	4.4±1.1 [bc]
	1 : 50	30	90	6	5.5±1.0 [bc]
		45	90	6	3.4±0.2 [c]
		60	90	4	3.3±0.1 [c]
Non-shaking	1 : 10	30	90	4	6.7±0.4 [b]
		45	90	4	5.6±1.1 [bc]
		60	90	4	4.4±1.0 [bc]
	1 : 50	30	90	3	3.4±0.2 [c]
		45	90	5	4.4±0.9 [bc]
		60	90	4	4.7±1.5 [bc]

Transformation efficiency was recorded as the percentage of PLBs that produced shoot primordia after 4 months of secondary selection on medium containing 20 mg l^{-1} hygromycin and 20 mg l^{-1} meropenem. [1)] Recorded 4 months after transfer onto secondary selection medium. [2)] Means of 3 replications with the same letters show significant difference as analyzed by Tukey's test at $p<0.05$ after arcsine transformation of the data.

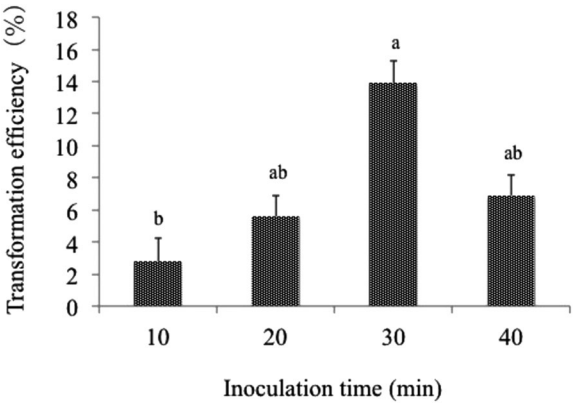

Figure 1. Effect of the inoculation time of PLBs with *Agrobacterium* on transformation efficiency of *Dendrobium* formidible 'Ugusu.' Means of 3 replications with the different letters show significant differences as analyzed by Tukey's test at $p<0.05$ after arcsine transformation of the data.

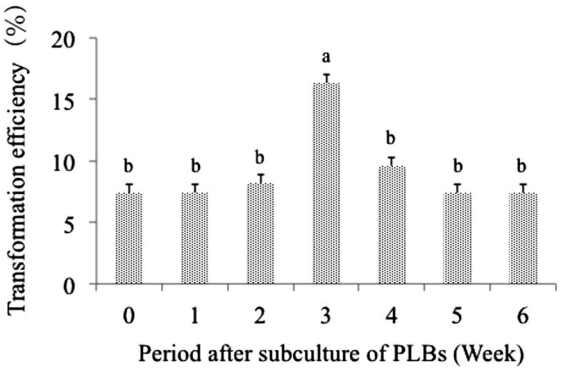

Figure 2. Effect of the age of PLBs on transformation efficiency of *Dendrobium* Formidible 'Ugusu.' Means of 3 replications with the same letters show significant difference as analyzed by Tukey's test at $p<0.05$ after arcsine transformation of the data.

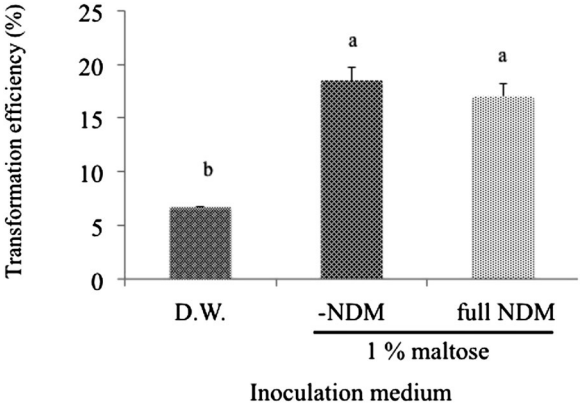

Figure 3. Effect of inoculation medium on transformation of *Dendrobium* Formidible 'Ugusu.' For each medium, 100 μM acetosyringone was added. Means of 3 replications with the different letters show significant difference as analyzed by Tukey's test at $p<0.05$ after arcsine transformation of the data.

Effect of different stage of PLBs after subculture and inoculation medium on transformation efficiency

In genetic transformation of various orchids, PLB has predominantly been used as target material for inoculation of *Agrobacterium* because it has high potential to regenerate into plants and could be easily obtained by culturing shoot tip (Martin et al. 2005; Shrestha et al. 2007; Tokuhara and Mii 1993) and root tip (Guo et al. 2010). In the present study, we investigated the suitable stage of PLBs for transformation. Among the 7 stages examined, PLBs obtained 3 weeks after subculture produced the highest transformation efficiency of 16.3% (Figure 2). It has been reported that transgene integration is favored in cells at the S- and G1-phase and target tissue in transformation must be actively dividing cells, which could be obtained by subculturing (Suwanaketchanatit et al. 2007). Julkifle et al. (2012) have reported that the cells in PLBs are actively dividing at the third week after subculture. Therefore, 3 week-old PLBs might be suitable as a target material for *Agrobacterium* infection.

The composition of inoculation medium has been known to influence the efficiency of transformation. In *Lilium*, Azadi et al. (2010) attempted to investigate the efficiency of transformation by removing some elements in Murashige and Skoog medium (MS) used for inoculation and co-cultivation. The highest transformation efficiency (25.4%) was obtained when calli were inoculated with *Agrobacterium* in MS medium lacking KH_2PO_4, NH_4NO_3, KNO_3 and $CaCl_2$. However, the inoculation medium with only distilled water (D.W.) and sucrose also showed high transformation efficiency of 10.7%. In the present study, medium containing only 10 gl^{-1} maltose gave slightly but not significantly higher transformation efficiency of 18.5% than that with normal ND medium (17.0%) (Figure 3), indicating that removal of ND mineral and organic components did not affect the transformation efficiency. In contrast, complete removal of medium components from the inoculation medium, i.e., only D. W., gave significantly lower transformation efficiency (6.7%), suggesting the importance of maltose during infection period for the successful transformation with *Agrobacterium*. Previous studies demonstrated that sugars greatly increased the expression of *vir* genes in combination with AS (Shimoda et al. 1990; Wise et al. 2005). In the present study, difference in the presence of maltose in medium was made only at short period of inoculation but not at subsequent cocultivation period. Therefore, effectiveness of maltose for *Agrobacterium* infection was exerted probably through its enhanced attachment to the host cells. For achieving more efficient transformation, it might be necessary to find out more suitable sugars for *Dendrobium* as shown in lily transformation (Azadi et al. 2010).

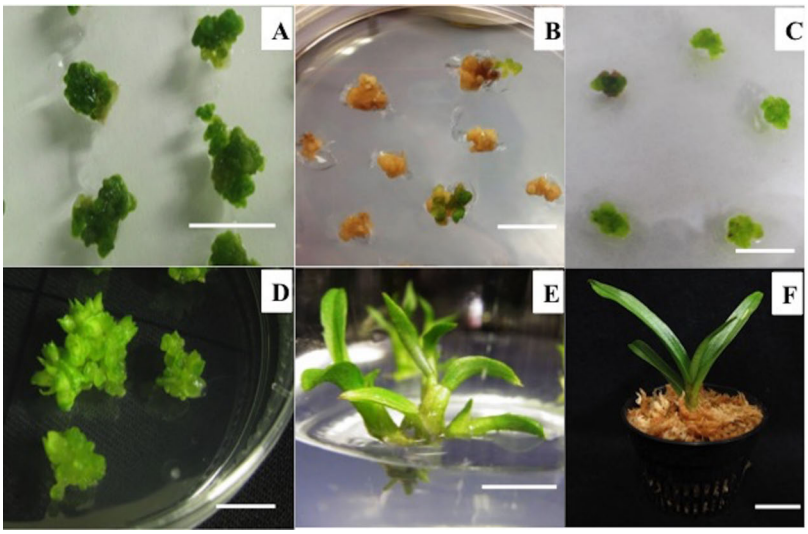

Figure 4. Plantlet regeneration of *Dendrobium* Formidible 'Ugusu' after infection of PLBs with *A. tumefaciens* (EHA101/pIG121Hm) and selection with hygromycin. A: PLBs after subculture for 3 weeks on ND medium containing 0.1 mgl^{-1} 1-naphthaleneacitic acid (NAA), 1.0 mgl^{-1} N^6-benzyladenine (BA), 1% maltose and 0.25% gellan gum at pH 5.4. B: Inoculated PLBs with *A. tumefaciens* (EHA101/pIG121Hm) after 6 weeks of culture on selection medium containing 20 mgl^{-1} hygromycin and 20 mgl^{-1} meropenem. C: Surviving PLBs after transfer to secondary selection medium containing 20 mgl^{-1} hygromycin and 20 mgl^{-1} meropenem for 1 month. D: Multiple shoots regenerated from secondary PLBs after secondary selection with 20 mgl^{-1} hygromycin for 4 months. E: Rooting plantlets regenerated from multiple shoots on selection medium with 20 mgl^{-1} hygromycin and 20 mgl^{-1} meropenem for 6 months. F: Successfully acclimatized transgenic plants grown for 3 months in a growth chamber at 25°C under a16-/8-h photoperiod. Bar=1 cm.

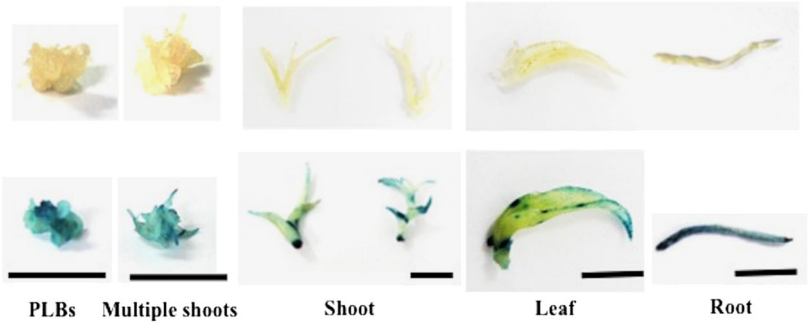

Figure 5. GUS histochemical assay of *Dendrobium* Formidible 'Ugusu' at different growth stages. PLBs: after selection on medium containing 20 mgl^{-1} hygromycin and 20 mgl^{-1} meropenem for 2 months. Multiple shoots: shoots regenerating from PLBs after secondary selection on medium 20 mgl^{-1} hygromycin and 20 mgl^{-1} meropenem for 3 months. Shoot: 5 months after regenerated from PLBs. Leaf and Root: 6 months after regeneration from PLBs. Un-transformed plant (upper) and transgenic plant (lower). Bar=1 cm.

Selection and regeneration of transgenic plants

When PLBs 3 weeks after subculture (Figure 4A) were cultivated for 2 months on the selection medium containing 20 mgl^{-1} hygromycin after co-cultivation (primary selection), some parts of them remained green (Figure 4B), whereas others turned brown and died. The survived PLB tissues were separated and cultured for one month on hygromycin-free ND medium on which they initiated to induce secondary PLBs. These PLBs with new proliferation were reselected by transferring and subculturing on the primary selection medium i.e. ND medium containing 20 mgl^{-1} hygromycin at one month intervals. One month after the transfer, all of the secondary PLBs survived (Figure 4C) and produced multiple shoots after 4 months of culture on medium (Figure 4D). When these multiple shoots were transferred onto medium containing reduced concentration of hygromycin to 10 mgl^{-1}, they produced roots and developed into plantlets with 3–4 leaves 6 months after the transfer (Figure 4E). These plantlets were successfully acclimatized in sphagnum moss of small pots in a growth chamber at 25°C under a 16-/8-h (day/night) photoperiod (Figure 4F).

GUS histochemical assay and confirmation of transgenic plants

Hygromycin resistant PLBs and multiple shoots showed histochemical GUS staining, which was not observed in untransformed control PLBs (Figure 5). GUS staining could also be detected in shoots, leaves and roots of the

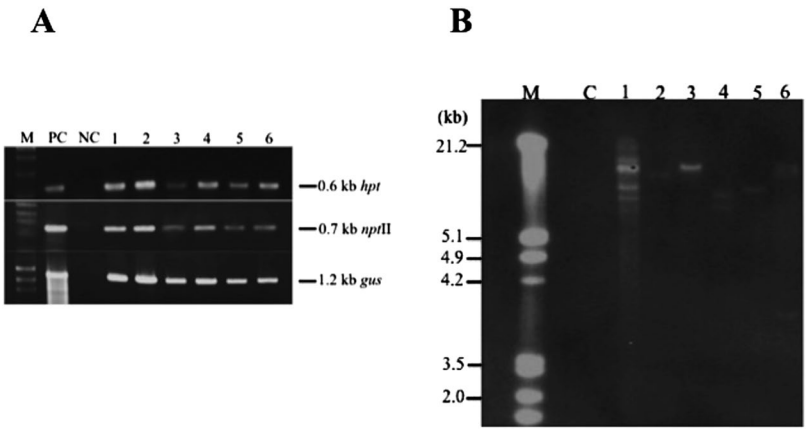

Figure 6. Molecular analysis of transgenic *Dendrobium* Formidible 'Ugusu.' (A) PCR analysis of transgenic plantlets for hygromycin phosphotransferase (*hpt*), neomycin phosphotransferase II (*nptII*) and β-glucuronidase (*gus*) genes. Lane M: Molecular size marker (φX174/*Hae*III), Lane PC: Plasmid pIG121Hm (positive control), Lane NC: untransformed plant as negative control (non-transformed plant), Lanes 1–6: Transgenic plants of *D.* Formidible 'Ugusu.' (B) Southern blot analysis of transgenic plants. Ten micrograms of genomic DNA was digested with *Hin*dIII and hybridized with digoxigenin (DIG)-labelled HPT gene probe. Lane M: Molecular size marker (DIG), Lane C: untransformed plant as negative control (non-transformed plant), Lanes 1–6: Transgenic plants.

regenerated plantlets. The presence of the *gus*, *nptII* and *hpt* genes in these putatively transformed plantlets was confirmed by PCR amplification, which showed the expected 1.2-, 0.7- and 0.6-kb amplification products, respectively (Figure 6A). Furthermore, Southern blot analysis using the *hpt* gene probe showed that all of the plantlets confirmed the presence of the *hpt* gene sequence in the genomes of all the 6 plantlets randomly selected. Of these six transgenic plantlets, 3 exhibited single insertion sites (Lanes 2, 3 and 5), whereas another 3 showed 2 (Lane 4) or more (Lanes 1 and 6) T-DNA insertion sites in their genomes (Lane 1) (Figure 6B).

In conclusion, we have succeeded in establishing an efficient transformation system for *D.* Formidible 'Ugusu' via *Agrobacterium*-mediated transformation. The factors optimized here, use of 3 week-old PLBs after regular subculture, appropriate inoculation medium (1% maltose+100 μM AS), and optimum bacterial concentration (OD_{600}=0.1) and inoculation time (30 min) could be applied for the transformation of various cultivars of *Dendrobium* and their hybrids. Introduction of the genes that regulate economically important traits such as flower color and disease resistance into *D.* Formidible 'Ugusu' are now in progress.

References

Azadi P, Chin DP, Kuroda K, Khan RS, Mii M (2010) Macro elements in inoculation and co-cultivation medium strongly affect the efficiency of *Agrobacterium*-mediated transformation in *Lilium*. *Plant Cell Tissue Organ Cult* 101: 201–209

Chang C, Chen YC, Hsu YH, Wu JT, Hu CC, Chang WC, Lin NS (2005) Transgenic resistance to *Cymbidium mosaic virus* in *Dendrobium* expressing the viral capsid protein gene. *Transgenic Res* 14: 41–46

Chia TF, Chan YS, Chua NH (1994) The firefly luciferase gene as a non-invasive reporter for *Dendrobium* transformation. *Plant J* 6: 441–446

Guo WL, Chang YC, Kao CY (2010) Protocorm-like bodies initiation from root tips of *Cyrtopodium paranaense* (Orchidaceae). *HortSci* 45: 1365–1368

Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J Bacteriol* 168: 1291–1301

Jefferson RA (1987) Assaying chimeric gene in plants: the GUS gene fusion system. *Plant Mol Biol Rep* 5: 387–405

Julkifle AL, Poobathy R, Samian R, Subramaniam S (2012) Histological analyses of PLBs of *Dendrobium* sonia-28 in the recognition of cell competence for regeneration and *Agrobacterium* infection. *Plant Omics J* 5: 514–517

Kuehnle AR, Sugii N (1992) Transformation of Dendrobium orchid using particle bombardment of protocorms. *Plant Cell Rep* 11: 484–488

Martin KP, Geevarghese J, Joseph D, Madassery J (2005) In vitro propagation of Dendrobium hybrids using flower stalk node explants. *Indian J Exp Biol* 43: 280–285

Men S, Ming X, Liu R, Wei C, Li Y (2003) *Agrobacterium*-mediated genetic transformation of a *Dendrobium* orchid. *Plant Cell Tissue Organ Cult* 75: 63–71

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* 8: 4321–4326

Ogawa Y, Mii M (2004) Screening for highly active β-lactam antibiotics against *Agrobacterium tumefaciens*. *Arch Microbiol* 181: 331–336

Ogawa Y, Mii M (2007) Meropenem and moxalactam: novel β-lactam antibiotics for efficient *Agrobacterium*-mediated transformation. *Plant Sci* 172: 564–572

Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol* 31: 805–813

Shimoda N, Toyoda-Yamamoto A, Nagamine J, Usami S, Katayama M, Sakagami Y, Machida Y (1990) Control of expression of

Agrobacterium *vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. *Proc Natl Acad Sci USA* 87: 6684–6688

Shrestha BR, Chin DP, Tokuhara K, Mii M (2007) Efficient production of transgenic plants of *Vanda* through sonication-assited *Agrobacterium*-mediated transformation of protocorm-like bodies. *Plant Biotechnol* 24: 429–434

Suwanaketchanatit C, Piluek J, Peyachoknagul S, Huehne PS (2007) High efficiency of stable genetic transformation in *Dendrobium* via microprojectile bombardment. *Biol Plant* 51: 720–727

Tee CS, Marziah M, Tan CS, Abdullah MP (2003) Evaluation of different promoters driving the GFP reporter gene and selected target tissues for particle bombardment of Dendrobium Sonia 17. *Plant Cell Rep* 21: 452–458

Tokuhara K, Mii M (1993) Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. *Plant Cell Rep* 13: 7–11

Wise AA, Voinov L, Binns AN (2005) Intersubunit complementation of sugar signal transduction in *Vir*A heterodimers and posttranslational regulation of VirA activity in *Agrobacterium tumefaciens. J Bacteriol* 187: 213–223

Yu H, Yang SH, Goh CJ (2001) *Agrobacterium*-mediated transformation of a *Dendrobium* orchid with the class 1 *knox* gene *DOH1. Plant Cell Rep* 20: 301–305

Zhang L, Chin DP, Mii M (2010) *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cattleya. Plant Cell Tissue Organ Cult* 103: 41–47

Zheng Q, Zheng YP, Wang GD, Guo WM, Fan EF, Wang C (2012) Sonication assisted *Agrobacterium*-mediated transformation of *ACC* gene to interfere the production of ethylene in Spring *Dendrobium* cv. 'Sanya.' *Russ J Plant Physiol* 59: 266–274