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Transgenic Note

Efficient production of transgenic plantls of *Vanda* through sonication-assisted *Agrobacterium*-mediated transformation of protocorm-like bodies

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Abstract In *Vanda* orchids, it is important to produce cultivars with economically important traits such as disease and pest resistances and novel flower colors, which are difficult to achieve by conventional cross breeding methods. To realize these breeding objectives, it is now expected to apply genetic transformation technology to introduce useful foreign genes into *Vanda* orchids. However, there has been almost no information on the genetic transformation of *Vanda*. Transgenic plants were successfully regenerated after co-cultivating protocorm-like bodies (PLBs) with *Agrobacterium tumefaciens* strain EHA101 (pIG121Hm) that harbored genes for β-glucuronidase (*gus*), hygromycin phosphotransferase (*hpt*) and neomycin phosphotransferase II (*nptII*). PLBs of 'Tokyo Blue' maintained in liquid New Dogashima medium (NDM) under dark condition, were subjected to transformation experiments. The PLBs inoculated with *Agrobacterium* produced secondary PLBs 4 weeks after transfer onto 3 g l^{-1} gellan gum-solidified NDM containing 30 g l^{-1} maltose, 10 mg l^{-1} meropenem and 10 mg l^{-1} hygromycin. Transformation efficiency was increased by prolonged period of infection (240 min) and wounding treatment of PLBs by sonication (5 min) during inoculation. Transformation of the hygromycin-resistant plantlets regenerated from different PLBs was confirmed by histochemical GUS assay, PCR analysis and Southern hybridization. With this transformation system, approximately 17 independent transgenic plants were obtained from 1 g PLBs.

Key words: *Agrobacterium tumefaciens*, genetic transformation, orchid, sonication, *Vanda*.

The genus *Vanda* comprises of about 35 species that are monopodial, mostly epiphytic and distributed mainly in tropical Asian regions. Because of the beautiful attractive flowers, they have been cultivated mainly in tropical countries and numerous cultivars have been produced through interspecific as well as intergeneric hybridization with allied genera such as *Aerides*, *Arachnis*, *Ascocentrum*, *Neofinetia*, *Renanthera* and *Rhynchostylis*. Although these breeding efforts have greatly increased the genetic variability of the vanda-type orchids, it is still desired to improve several important characters such as resistances to various diseases and pests and tolerances to environmental stresses such as low temperatures and low light intensities. Especially in *Vanda*, various diseases such as bacterial soft rot caused by *Erwinia carotovora*, shoot rot caused by *Phytophthora parasitica*, stem rot caused by *Sclerotium rolfsii*, leaf blight caused by *Colletotrichum gloeosporioides* are known to cause great losses to the orchid growers in unmarketable products (Duff and Daly 2002). It has recently been reported that integration and over expression of anti-microbial peptide genes such as ferredoxin-like protein gene and defensin gene in *Phalaenopsis* orchids resulted in the strong resistance to *Erwinia carotovora* (Chan et al. 2005; Sjahril et al. 2006). Although transformation of orchids has been achieved through particle bombardment in several orchids such as *Phalaenopsis* (Anzai et al. 1996), *Dendrobium* (Kuehnle and Sugii 1992; Chia et al. 1994), *Brassia*, *Cattleya* and *Doritaenopsis* (Knapp et al. 2000) and through *Agrobacterium*-mediated transformation in *Phalaenopsis* (Belarmino and Mii 2000; Chai et al. 2002; Mishiba et al. 2005; Sjahril and Mii 2006), *Oncidium* (Liau et al. 2003) and *Dendrobium* (Men et al. 2003), there has been only one brief report on the transformation of *Vanda* (Chia et al. 1990), via particle bombardment and no reliable protocol for the genetic transformation of *Vanda* has been established.

In this paper, we report the successful results on the

Abbreviations: AS, acetosyringone; BA, benzyladenine; GUS, β-glucuronidase; NAA, α-naphthaleneacetic acid; NDM, New Dogashima medium; PLB, protocorm-like body

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production of transgenic plantlets through sonication-assisted *Agrobacterium*-mediated transformation of protocorm-like bodies (PLBs) in *Vanda*.

Protocorm-like bodies (PLBs) induced from shoot tip explants of *Vanda* 'Tokyo Blue' were used in the transformation study. Since it is rather difficult to obtain enough number of shoot tip explants from each plant of *Vanda* because of its monopodial nature, they were obtained from the secondary shoots of ca. 10 cm long induced after pinching the apex of the main shoot. The method of shoot tip culture was almost the same as that previously described for *Phalaenopsis* (Tokuhara and Mii 1993). After disinfecting the apical part (5 cm long) of the secondary shoots for 10 min with 5% $Ca(ClO)_2$ solution containing a few drops of Tween 20, the leaves were removed and the shoot tip explants (5 mm height×3 mm width) were excised with a forceps and placed onto 2 g l^{-1} gellan gum-solidified New Dogashima medium (NDM) (Tokuhara and Mii 1993) containing 10 g l^{-1} sucrose supplemented with 0.1 mg l^{-1} NAA and 1 mg l^{-1}BA (pH 5.4). They were then cultured at 23°C under 14 h photoperiods provided by fluorescent lamps (33 μmol m^{-2} s^{-1}). PLBs formed were sub-cultured onto 3 g l^{-1} gellan gum-solidified NDM supplemented with 10 g l^{-1} sucrose, maltose or lactose and the same concentrations of NAA and BA. PLBs were routinely sub-cultured every 4 weeks by transferring 0.5 g PLBs into 40 ml liquid NDM containing 10, 30, 60 or 90 g l^{-1} maltose supplemented with 0.1 mg l^{-1} NAA and 1 mg l^{-1} BA (pH 5.4) in a 100 ml flask. The cultures were kept on a reciprocal shaker at 75 rpm under the dark conditions at 25°C with the relative humidity of 55–75%.

Agrobacterium tumefaciens strain EHA101 (Hood et al. 1986) harboring the binary vector plasmid pIG121Hm (Ohta et al. 1990), which contains a hygromycin phosphotransferase gene (*hpt*) and a neomycin phosphotransferase II gene (*nptII*) as selectable markers and an intron-containing β-glucuronidase (*gus*) reporter gene, was used for transformation.

Two days before *Agrobacterium* infection, the PLBs were pre-cultured by transferring into fresh liquid NDM supplemented with 0.1 mg l^{-1} NAA and 1 mg l^{-1} BA. Three kinds of wounding treatments were applied for PLBs before the infection to increase the transformation efficiency. 1) Slicing: The PLBs were wounded by slicing into 3–4 mm thick explants when they were pre-cultured 2 days before inoculation into fresh medium, to which 100 μM acetosyringone (AS) was added. 2) Scratching: The surface of the PLBs was wounded by using sandpaper (grit number 150, Fuchioka, Japan) attached to the inner surface of plastic centrifuge tubes (50 ml; Iwaki, Japan) (Hoshi et al. 2004). The PLBs were transferred into the tubes containing 15 ml liquid NDM and stirred using an automatic lab-mixer (Ikeda Scientific Co., Ltd, Japan) for 30 s. 3) Sonication: PLBs were placed in a mixture of 30 ml liquid NDM and *Agrobacterium* liquid culture (OD_{600}=0.6) at 10 : 1 ratio and subjected to sonication treatment for 5 min at 80% ultrasound power (Transsonic Digital S, Elma, Germany).

A. tumefaciens was grown overnight at 28°C in Luria-Bertani (LB) medium containing 50 mg l^{-1} hygromycin, 50 mg l^{-1} kanamycin and 25 mg l^{-1} chloramphenicol, adjusted to pH 7.2. For inoculation, 3 ml of *Agrobacterium* liquid culture was added to 30 ml of PLB culture containing 1 g PLBs (approx. 25 PLBs) to give a bacterial density of OD_{600}=0.5, which was incubated for 10 min to 480 min at 25°C. The PLBs were collected on a stainless steel mesh and blotted dry before transferring onto 3 g l^{-1} gellan gum-solidified NDM supplemented with 0.1 mg l^{-1} NAA, 1 mg l^{-1} BA, 30 g l^{-1} maltose and 0, 100, 200 or 400 μM AS for co-cultivation. After 3 days of co-cultivation, the PLBs were placed for 2 weeks on the same NDM but containing 5 mg l^{-1} meropenem (Meropen®; Sumitomo Pharmaceutical Ind. Co. Ltd., Osaka, Japan), which is highly effective for eliminating *Agrobacterium* without serious toxic effects on plant tissues (Ogawa and Mii 2005, 2007; Sjahril and Mii 2006; Chin et al. 2007). For selection, the PLBs were transferred onto 3 g l^{-1} gellan gum-solidified NDM containing 30 g l^{-1} maltose, 10 mg l^{-1} meropenem and 10 mg l^{-1} hygromycin and cultured for 8 weeks by sub-culturing at 2 week-intervals onto fresh medium with the same composition. PLBs that had grown and retained their green color on the selection medium were cut transversely into 2 pieces (2–3 mm) and placed for the growth recovery on the same medium but lacking hygromycin (recovery medium) for 1–2 months. The newly proliferated PLBs were then returned to the selection medium containing 10 mg l^{-1} hygromycin.

PLBs growing on 10 mg l^{-1} hygromycin containing medium were selected as putative transformants and transferred onto a hormone-free (HF) regeneration medium which was 3 g l^{-1} gellan gum-solidified NDM containing 10 g l^{-1} maltose, 10 mg l^{-1} meropenem and 10 mg l^{-1} hygromycin. Regenerated plantlets were further transferred onto a hormone-free (HF) regeneration medium in culture bottles (length, 13 cm; diameter, 7 cm) under a 16 h photoperiod (35 μmol m^{-2} s^{-1}) at 25°C with a relative humidity of 55–75%.

Stable expression of β-glucuronidase (GUS) in hygromycin-resistant PLBs, and leaves and roots of hygromycin-resistant plants was observed by histochemical staining with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) (Jefferson et al. 1987). Tissues were incubated in a solution containing 50 mM phosphate buffer, 1 mg ml^{-1} X-gluc and 0.5% (v/v) Triton X-100, aspirated in a desiccator for 10 min to infiltrate the solution into the plant tissues, and incubated overnight at 37°C. After the staining, PLBs and plantlets were soaked in 70%

ethanol to remove the chlorophyll.

Ten microgram of total genomic DNA was extracted from a non-transformed plant and putative transgenic plantlets derived from different transgenic PLB lines, using the cetyl-trimethyl-ammonium bromide (CTAB) method (Murray and Thompson, 1980). PCR detection of the transgenes was performed as described by Hamill et al. (1991) for *gus* and *nptII* and Xiao and Ha (1997) for *hpt*, respectively. PCR was performed under the following conditions: 30 cycles of 1 min at 94°C, 1 min at 62°C (55°C for *hpt*, 59°C for *nptII*) and 1.5 min at 72°C.

For Southern blot analysis, the genomic DNA (10 μg) from hygromycin-resistant transgenic plantlets and control plantlet was digested with *Hin*dIII and fractionated through a 0.8% (w/v) agarose gel by electrophoresis, blotted to a membrane (Immobilon-NY$^+$, Millipore, Bedford, MA, USA) and hybridized with digoxigenin-labelled *gus* gene probe following the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

PLBs sub-cultured on medium containing 10 g l^{-1} maltose showed higher proliferation rate and less occurrence of necrosis than those cultured on medium containing the same concentration of sucrose or lactose and gave 6-fold increase in fresh weight after 45 days of culture when 0.2 g PLBs were inoculated (Table 1). Further study revealed that optimum concentration of maltose for multiplication of PLBs was 30 g l^{-1}, which gave 6-fold increase in fresh weight after 4 weeks of culture. Therefore, *Vanda* PLBs sub-cultured on medium with 30 g l^{-1} maltose at 4-week intervals were used for the transformation in further experiments.

Although the plasmid used in this study (pIG121Hm) contains both *nptII* and *hpt* genes as selectable markers, kanamycin failed to inhibit the growth even at 400 mg l^{-1} after 1 month of culture in our preliminary experiment. In contrast, hygromycin showed high toxicity to PLBs of *Vanda* and 10 mg l^{-1} hygromycin completely inhibited the growth of PLBs and caused necrosis after 1 month of treatment. Consequently, we used 10 mg l^{-1} hygromycin for selection of putative transformants.

In the present study, the inoculation period significantly affected the production of hygromycin resistant PLBs and the highest efficiency of transformation was obtained when 240 min inoculation period was applied, namely ca. 5 hygromycin-resistant PLBs were obtained from 1 g PLBs infected (Figure 1). However, inoculation for 480 min was toxic to the PLBs of *Vanda* and caused necrosis. Positive effect of long infection period has also been reported in other orchids such as *Phalaenopsis* (Belarmino and Mii 2000; Mishiba et al. 2005) and *Cymbidium* (Chin et al. 2007). Therefore, it is possible that orchids generally require the prolonged infection period to ensure the tight adhesion of bacteria onto the surface of PLBs.

Table 1. Effect of several carbohydrates on growth of PLBs in *Vanda*

Carbohydrate (10 g l^{-1})	PLB (g)		
	Green	Brown	Total
Sucrose	0.52±0.09	0.19±0.03	0.71±0.08
Maltose	1.16±0.12	0.06±0.01	1.22±0.11
Lactose	0.21±0.07	0.21±0.02	0.42±0.06

0.2 g PLBs were inoculated onto 3 g l^{-1} gellan gum-solidified NDM supplemented with 0.1 mg l^{-1} NAA, 1 mg l^{-1} BA and 10 g l^{-1} of each carbohydrate, and fresh weights of green PLBs and necrotic part were measured after 45 days of culture. Each value represents a mean±SE of the three independent experiments.

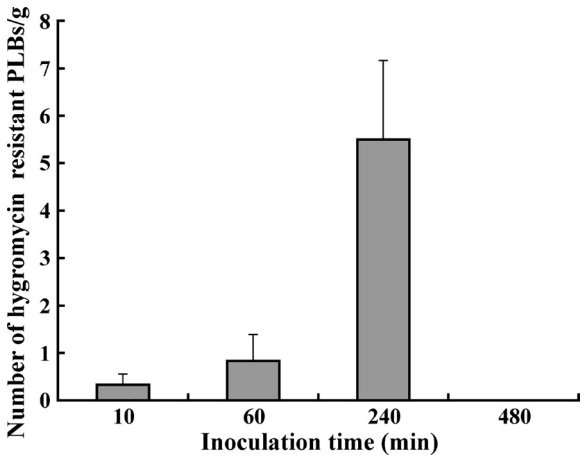

Figure 1. Effect of inoculation period of *Agrobacterium* on the production of hygromycin-resistant PLBs of *Vanda*. Two g of PLBs were used as explants and the number of independent secondary PLBs produced on the hygromycin-containing medium was scored 3 months after the bacterial inoculation. The values represent the average with SE (vertical bar) of three independent experiments.

In *Agrobacterium*-mediated transformation, wounding treatments such as sonication (Trick and Finer 1997, 1998; Amoah et al. 2001; Tang 2003; Zaragoza et al. 2004; Gaba et al. 2006) and scratching (Hoshi et al. 2005) have been used to enhance transformation in various species. In the present study, all of the 3 kinds of wounding treatments increased the transformation efficiency by 2–5 folds in comparison with the control and the highest transformation efficiency was obtained by sonication treatment, which gave ca. 17 independent transgenic PLBs per 1 g explants used for inoculation (Figure 2). The enhanced transformation rates using sonication probably resulted from micro wounding (Gaba et al. 2006), which might have been more favorable for *Agrobacterium* infection compared with the other two wounding treatments which might have given large scale and deep damage on the explants.

In *Agrobacterium*-mediated transformation, pre-culture of the explants before inoculation sometimes resulted in high transformation efficiencies (Jacq et al. 1993; Robichon et al. 1995; Mishiba et al. 2005). In the present study, however, the number of hygromycin

resistant PLBs did not increase significantly by applying AS up to 400 μM in both infection and co-cultivation media (data not shown).

In our preliminary experiment, we found that all of the PLBs turned brown if they were directly cultured on selection medium containing 10 mg l^{-1} hygromycin. In contrast, when the *Agrobacterium*-inoculated PLBs were cultured on the selection medium after 2 weeks of culture on recovery medium, a few PLBs eventually produced compact yellow calli after turning brown within 3–4 weeks (Figure 3A). These yellow calli turned to be green PLBs during the subsequent subcultures on the same medium (Figure 3B). The green PLBs were then cut transversely and placed on recovery medium again for 1–2 months for producing secondary PLBs, which were further transferred onto selection medium and finally onto regeneration medium (Figure 3C). Consequently, all of the hygromycin resistant PLBs successfully developed into plantlets 1 year after inoculation with *A. tumefaciens* (Figure 3D). In contrast, the cut green PLBs showed considerable delay in producing secondary PLBs when they were directly transferred onto selection medium.

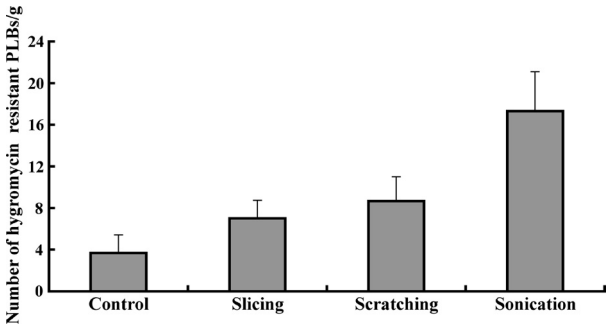

Figure 2. Effect of three wounding treatments: slicing, scratching and sonication treatments, on the transformation of *Vanda* PLBs. For each treatment, 1 g of PLBs were inoculated with *Agrobacterium* at a density of OD_{600}=0.5 for 240 min with 100 μM acetosyringone, co-cultivated for 3 days, and cultured on recovery medium for 2 weeks and selection medium thereafter. Transformation efficiency was evaluated by scoring the number of hygromycin-resistant PLBs 3 months after the bacterial inoculation. The values represent the average of 3 independent experiments.

Histochemical GUS staining was observed in all the hygromycin-resistant PLBs and was not observed in untransformed control PLBs. The PLBs, whole leaves and the roots of the hygromycin-resistant putative transformants were completely stained blue (Figure 3E–H), suggesting that reselection procedure had excluded possible chimeras. PCR analysis showed that all of the 12 randomly selected putative transformants had positive bands of the *hpt* gene, indicating that the present transformation procedure achieved strict selection of positive transformants. The presence of the *gus* and *nptII* genes was also confirmed by PCR amplification of a 1.2- and 0.7-Kb fragment in all the DNAs extracted from the putative transformants except one plant which lacked a

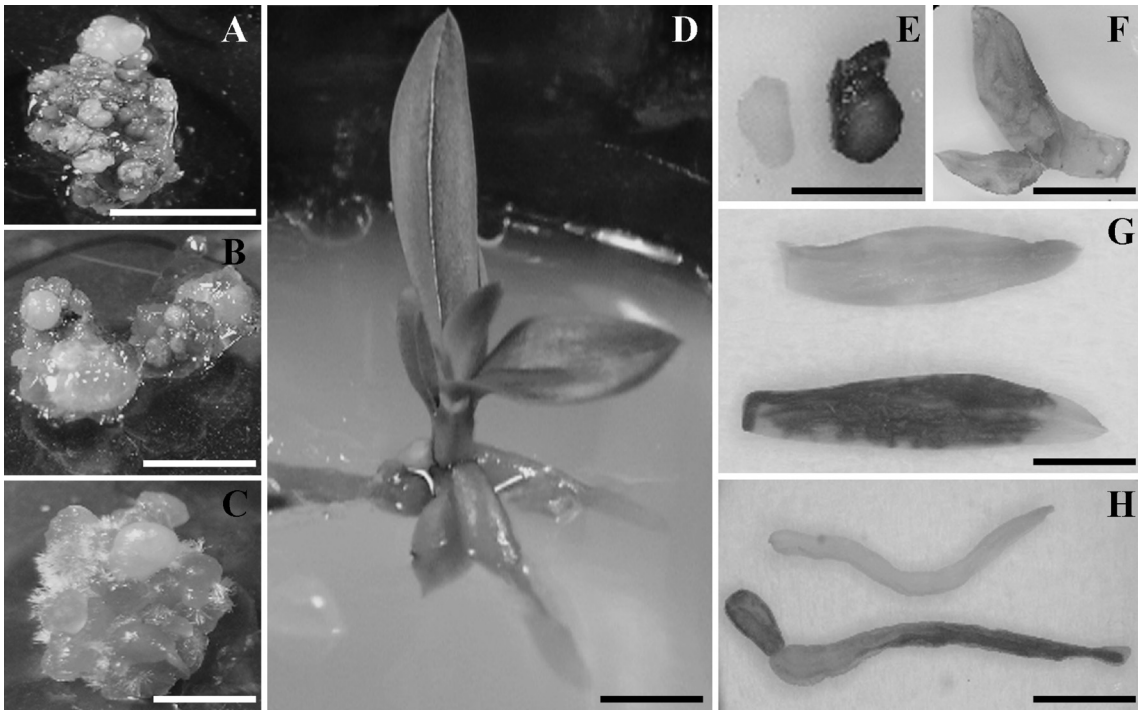

Figure 3. Regeneration of transgenic plant of *Vanda* 'Tokyo Blue'. (A) Emergence of putative transgenic compact yellowish callus from browned compact callus on selection medium. (B) PLBs formed 4 months after inoculation of *Agrobacterium* on medium containing 10 mg l^{-1} hygromycin. (C) Proliferation of PLBs in regeneration medium, (D) transgenic plantlet of *Vanda* 12 months after inoculation. (E) Transgenic PLB of *Vanda* showing blue staining of GUS (right) and control (left). (F) Transgenic shoot with GUS expression. (G) leaf and (H) root showing GUS expression (bottom) and the controls (top). Bar=5 mm.

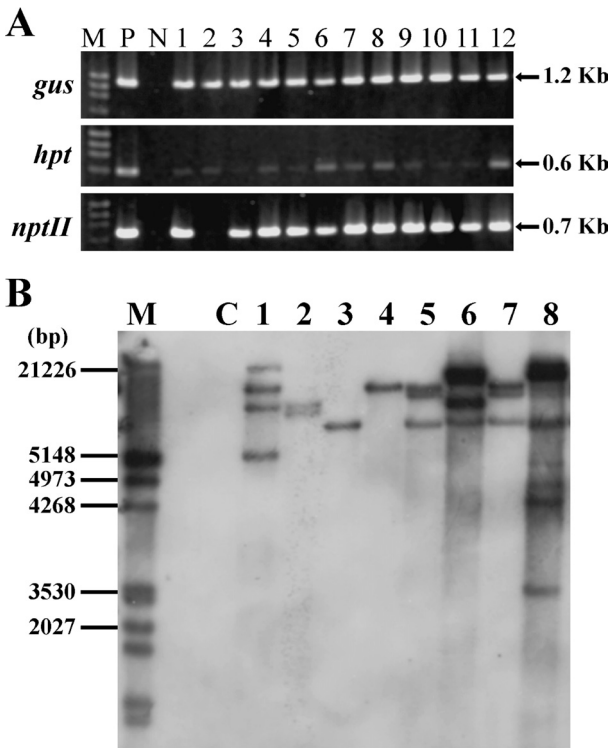

Figure 4. Molecular analysis of transgenic plants. (A) PCR analysis of transgenic *Vanda* plantlets for the hygromycin phosphotransferase (*hpt*), β-glucuronidase (*gus*) and neomycin phosphotransfease (*nptII*) genes. Lanes: *M* DNA size markers (ϕX174/*Hae* III) (1,353; 1.078; 872 and 603 bp fragments from top to bottom); *P* plasmid pIG121Hm (positive control); *N* non-transformed plant as control; *1–12* transgenic plantlets of *Vanda*. Arrows: Positions of the expected 1.2-, 0.6- and 0.7-Kb fragments for *gus*, *hpt* and *nptII* 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: *M* molecular marker, *C* non-transformed plant, *1–8* transgenic plantlets.

band of *nptII*, indicating that T-DNA integration had been accomplished normally in most cases (Figure 4A). Furthermore, southern blot analysis on eight randomly selected hygromycin-resistant plantlets revealed that all the plantlets confirmed the presence of the *gus* gene sequences in the plant genomes. Of the eight transgenic plantlets analyzed, 2 exhibited single insertion sites, whereas the rest showed 2 or more (Figure 4B).

We have successfully established for the first time, a simple and reliable procedure for *Agrobacterium tumefaciens*-mediated transformation of *Vanda* with sonication treatment, which gave ca. 17 independent transgenic plants from 1 g PLBs used for inoculation. The transformation system for *Vanda* established in this study will permit the introduction of genes that regulate morphological and economically important traits such as flower color, size and resistance to insects and diseases. Moreover, transgenic *Vanda* plants will be used as the source of protoplasts, which enable simple and reliable selection of the somatic hybrids in inter-generic and inter-specific hybrid production. Finally, the introduction of other genes such as defensin gene, which confers resistance to *Erwinia carotovora* (Sjahril et al. 2006), and green fluorescent protein (GFP) gene is now in progress.

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