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High efficiency of stable genetic transformation in *Dendrobium via* microprojectile bombardment

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Abstract

Several parameters affecting transient β-glucuronidase (GUS) expression in protocorms of *Dendrobium* cv. Jaquelyn Thomas were examined using bombardment technique with pActin-1D plasmid. The GUS activity in *Dendrobium* protocorm was not significantly affected by size of the target, type of particles, and helium gas pressure. However, the numbers of surviving tissues after bombardment were different. Transgenic orchids were established by bombardment of actively dividing protocorms with gold particles coated with pMNK1005 plasmid containing hygromycin phosphotransferase (*hgh*) and green fluorescent protein (*gfp*) genes driven by a ubiquitin promoter. A high efficiency of orchid transformation was established using three selection steps. The bombarded protocorms were screened on medium supplemented with 5 mg dm^{-3} and 25 mg dm^{-3} hygromycin and surviving protocorms were stringently selected on medium containing 30 mg dm^{-3} hygromycin. The transformation efficiency was 19.87 % and GFP expressing protocorms were not chimeras. The integration of the transgene into genomic DNA of transgenic plantlets was confirmed by PCR and Southern blot hybridization. All but one of the transgenic lines contained multiple copies of the transgene.

Additional key words: β-glucuronidase, green fluorescent protein, orchid, PCR, Southern blot.

Introduction

Orchid is one of the most attractive ornamental plants in the world because of its beauty and diversity. The potted plants and flowers of the orchid *Dendrobium,* in particular, have been in great demand and are valuable agricultural products on the global markets. Breeders, therefore, have been attempting to produce new variant cultivars that carry desirable traits, such as: novel shapes and colours, longer vase life, and resistance to pests and diseases (Liau *et al.* 2003). Because orchids have long reproduction cycle, slow seed maturation and poor fertility, conventional breeding techniques are of limited value in the improvement of orchid. Hence, the use of genetic engineering technique to introduce foreign genes into target cells is a promising alternative for orchid genetic improvement. So far, there have been remarkable reports in the development and establishment of transgenic orchids through bombardment transformation, such as for *Dendrobium* (Kuehnle and Sugii 1992, Chia *et al.* 1994, Nan and Kuehnle 1995, Yu *et al.* 1999, Men *et al.* 2003, Tee *et al.* 2003), *Phalaenopsis* (Anzai *et al.* 1996, Liao *et al.* 2004), *Cymbidium* (Yang *et al.* 1999), *Brassia, Cattleya, Doritaenopsis* (Knapp *et al.* 2000), and *Oncidium* (You *et al.* 2003), and using *Agrobacterium*-mediated gene delivery, such as for *Phalaenopsis* (Belarmino and Mii 2000, Chai *et al.* 2002), *Dendrobium* (Yu *et al.* 2001) and *Oncidium* (Liau *et al.* 2003).

However, the use of a genetic engineering technique has to incorporate at least two essential genes, a reporter gene and a selectable marker, into the plant cell. The reporter genes are visible markers, which can be used to investigate foreign gene expression in transformed cells. Many reporter genes have been utilized for plant transformation including β-glucuronidase (*gus*) gene, the anthocyanin pigmentation gene, the firefly luciferase

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Abbreviations: GFP - green fluorescent protein; GUS -β-glucuronidase; VW - Vacin and Went medium.

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gene, and the green fluorescent protein (*gfp*) gene. The *gfp* gene is a particularly useful potential marker due to its having a non-destructive assay, which no required substrate or key enzymes for screening and further analysis. Since this reporter system has many advantages, it is widely used for both monocot and dicot plants. In orchid, the *gfp* gene was recently used as a reporter in *Dendrobium* Sonia 17 transformation through micro-projectile bombardment (Tee *et al.* 2003).

Another important gene is the selectable marker, which can be used to eliminate untransformed cells during selection. For orchid transformation, several selectable marker genes for resistance to antibiotics or herbicides which serve as selective agents have been utilized, including neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPH), and phosphinotricin acetyltransferase (BAR). Since orchid cells are normally not sensitive to any selective agents, it is necessary to use a highly concentrated agent for the selection. The high concentration of gentamicin (450 - 600 mg dm^{-3}) or kanamycin (500 - 600 mg dm^{-3}) used to eliminate untransformed calli in various species of *Dendrobium* orchid might cause the occurrence of chimera tissues (Chia *et al.* 1994). Long-term use of a low dosage of kanamycin (50 - 100 mg dm^{-3}) for selection of transgenic *Dendrobium* orchid could not completely eliminate un-bombarded tissues (Griesbash 1994, Nan and Kuehnle 1995). Moreover, these antibiotics inhibited the regeneration of putative transgenic tissues to plantlets (Kuehnle and Sugii 1992). Accordingly, it is difficult to use this selection strategy to recover the orchid transgenic. On the other hand, hygromycin is a potential selective agent in discriminating transformed tissues from untransformed tissues that is widely used to produce transformed orchids (Yu *et al.* 1999, Belarmino *et al.* 2000, Men *et al.* 2003, Chai *et al.* 2002, Liau *et al.* 2003, You *et al.* 2003, Liao *et al.* 2004). However, the high concentration of hygromycin that is necessary might affect the life and the regeneration of transformed protocorms, the selection process is still a difficult step in the effective orchid's genetic transformation.

This research is focused on the selection step for stable genetic transformation in orchid by relying on the functions of both selectable and reporter genes. In this communication, dividing *Dendrobium* orchid protocorms were bombarded with vector containing the *hph* and *gfp* genes and cells expressing *gfp* were identified under fluorescence microscope during the selection process. The report provides a new technique for orchid transformation with high efficiency establishing of non-chimera orchid tissues.

Materials and methods

Plants and culture conditions: A green capsule of *Dendrobium* Jaquelyn Thomas obtained from Rapee Sagarik Orchid Garden at Kasetsart, Bangkok, University, Thailand was immersed in 70 % ethanol and rinsed three times with sterile distilled water. The seeds were taken out from sterilized capsule and primary protocorms were produced by culturing in solid Vacin and Went (1949) (VW) medium supplemented with 20 g dm^{-3} sucrose, 100 g dm^{-3} homogenized ripe banana, 100 g dm^{-3} homogenized potato, 150 cm^3 dm^{-3} coconut water, 4 g dm^{-3}charcoal, and 7 g dm^{-3} agar. These primary protocorms were proliferated to secondary protocorms in 30 cm^3 of liquid ½ MS (Murashige and Skoog 1962) medium containing 15 g dm^{-3} sucrose and 75 cm^3 dm^{-3} coconut water by incubating them with shaking at 100 s^{-1} and sub-culturing twice a week. 7 d prior to transformation, these tissues were sub-cultured to produce newly active protocorms, which would be used as a target tissue for bombardment.

Plasmids: The pActin1-D plasmid (kindly provided by Dr. R. Wu) containing β-glucuronidase (*gus*) gene driven from an Act-1 promoter and Nos terminator was used to determine the parameters for bombardment of protocorms by observation of transient GUS expression. The pMNK1005 plasmid (Upadhyaya *et al.* 1998) containing a translational fusion of the hygromycin phosphotransferase (*hph*) gene and the green fluorescent protein (*S65Tgfp*) gene expressed under Ubi-1 promoter and Nos terminator, was used as a cassette to assess stable genetic transformation in *Dendrobium* orchid.

Microprojectile bombardments: 4 h before bombardment, protocorms were placed in a circle with a diameter of 25 mm on solid MS medium supplemented with 30 g dm^{-3} sucrose, 8 g dm^{-3} mannitol, and 7 g dm^{-3} agar for osmotic pretreatment. The plasmid coated microparticles (Christou *et al.* 1991) were delivered into the protocorm with the distance from macrocarrier to target tissue set at 90 mm and vacuum chamber set at 84 kPa using a *Biolistic PDS-1000/Helium* (*Bio-Rad Laboratories*, Hercules, CA, USA). To assess the efficiency of transient GUS expression, the pActin-1D plasmid was coated onto two types of particle, gold (1.0 μm in diameter from *Bio-Rad*) and tungsten (1.0 - 1.3 μm in diameter from *Bio-Rad*) with two levels of helium gas pressure, 6 200 and 7 500 kPa. Two different sizes of target tissue, approximately 1 - 2 mm and 3 - 5 mm in diameter were evaluated to determine the appropriate size of protocorm for orchid transformation. After bombardment, bombarded tissues were maintained in the dark growth chamber for 3 d before culturing in solid MS medium under a 16 h photoperiod with cool white light at 25 °C. A suitable parameter condition subsequently was used to establish transgenic orchids with the pMNK1005 plasmid.

GUS histochemical assay: The GUS in protocorms activity was examined in 3 d after bombardment using histochemical staining (Jefferson *et al.* 1987). The bombarded protocorms were immersed in 5-bromo-4-chloro-3-indonyl-B-D-glucuronic acid (X-gluc) buffer containing 1 mM X-gluc, 100 mM sodium phosphate buffer, pH 8.0, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide and incubated overnight at 37 °C. After staining, blue spots appeared in the cells expressing *gus* gene. The chlorophyll content in the bombarded tissues was removed with 70 % ethanol in order to examine and count the blue spots that occurred on the protocorms.

Selection of transformants: 3 d after bombardment, transformed tissues were initially selected in solid MS medium supplemented with 5 mg dm^{-3} hygromycin for 45 d. Proliferating protocorms were chopped and transferred to solid MS medium supplemented with 25 mg dm^{-3} hygromycin. The green tissues were maintained in 25 mg dm^{-3} of selective media for 45 d and subsequently selected in solid MS medium supplemented with 30 mg dm^{-3} hygromycin for another 30 d. Control explants were also selected in the same way. Later, the hygromycin-resistant protocorms were transferred to solid MS medium without selective agent for regeneration of plantlets.

GFP detection: The bombarded protocorms having been in selective media for 7, 45, 90, or 120 d were observed for their GFP expression with a fluorescence microscope (*Nikon E600*, Tokyo, Japan) equipped with a *BZA* filter (*Nikon*), and the images were captured in real time with a *Nikon Coolpix5400* camera attached to the fluorescence microscope using *Nikon* software.

PCR analysis and Southern blot hybridization: Genomic DNA from fresh leaves of individual transgenic lines and un-bombarded plants were extracted using the CTAB method (Murray and Thomson 1980) and used for amplification of internal *hph* and *gfp* genes. The thermal cycling program was performed as follows: 35 cycles of 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C. The PCR products were hybridized with *hph* and *gfp* gene probes to confirm the identity of the 600 bp fragment of the *hph* gene and the 800 bp of the *gfp* gene. The pMNK1005 plasmid was digested with *Nco*I to produce the internal *hph* and *gfp* fragments labeled using *Gene images Alkphos Direct* labelling (*Amersham Bioscience*, Buckinghamshire, UK).

The genomic DNA from independent transgenic lines and un-bombarded plants were used to confirm the integration of the *gfp* gene in chromosomes of the transgenic orchid. 10 µg of genomic DNA digested with *Sac*I were separated in a 1 % of agarose gel and blotted onto *Hybond* N^+ nylon membrane (*Amersham Bioscience*) using capillary blotting with 10× SSC. The blotted membrane was hybridized at 60 °C with the *gfp* probe. After hybridization, the membrane was washed twice with 1st washing buffer (2 M urea, 1 g dm^{-3} SDS, 50 mM NaH_2PO_4, 150 mM NaCl, 1 mM $MgCl_2$, and 2 g dm^{-3} blocking reagent) for 10 min at 60 °C, and then re-washed with 2nd buffer (1 mM Tris base, 2 M NaCl, and 2 mM $MgCl_2$) twice at room temperature. The signal was detected using CDP star substrate (*Amersham Bioscience*) and exposed the signal on X-ray film (*Kodak*) at room temperature.

Results and discussion

Effective parameters for transient GUS expression: To determine the optimal condition for particle bombardment, the transient GUS activity of transformed *Dendrobium* orchids was examined, comparing types of particle, gold and tungsten, helium gas pressure, and sizes of target tissue, small size (1 - 2 mm in diameter) and large size (3-5 mm in diameter). Transient GUS expression was observed in the secondary protocorms after bombardment with pActin-1D plasmid carrying the *gus* gene driven by Act-1 promoter and Nos terminator. The initial observation found that the range from macrocarrier to target tissue had a significant effect on the death of bombarded tissue. The target distance of 60 mm greatly damaged the bombarded small tissue, such that 99.66 % of bombarded small protocorms were dead, while the distance of 90 mm caused only 14.12 % to die (data not shown). Furthermore, the blue spots on protocorms with the flight distance of 90 mm (Fig. 1*B*) were more spreader-out than 60 mm (Fig. 1*A*). The target distance of 90 mm, therefore, was a suitable parameter to be used for further orchid transformation.

No significant transient GUS expression of protocorms bombarded with different microparticles was found. The number of GUS spots per protocorm after bombardment with gold particles, 1.0 µm in diameter from *Bio-Rad*, and tungsten particles, 1.0 - 1.3 µm in diameter from *Bio-Rad*, were 28.16 and 23.44, respectively (Table 1). The round surfaced spherical gold particles produced well spread blue spots (Fig. 1*C*), while the irregular surfaced of sharp tungsten particles gave a large blue patch (Fig. 1*D*) because of the aggregation during DNA coating. Furthermore, since the sharpness and toxicity of tungsten particles also damaged tissues (Hunold *et al.* 1994, Sanford *et al.* 1993), the number of surviving tissues after bombardment with gold was more than with tungsten (Table 1). Russell *et al.* (1992) showed no difference between tungsten and gold particles on the transformation rate of tobacco, but the rate of stable transformation with gold was four times higher than that of tungsten; consequently, gold particles are remarkably more efficient for genetic transformation.

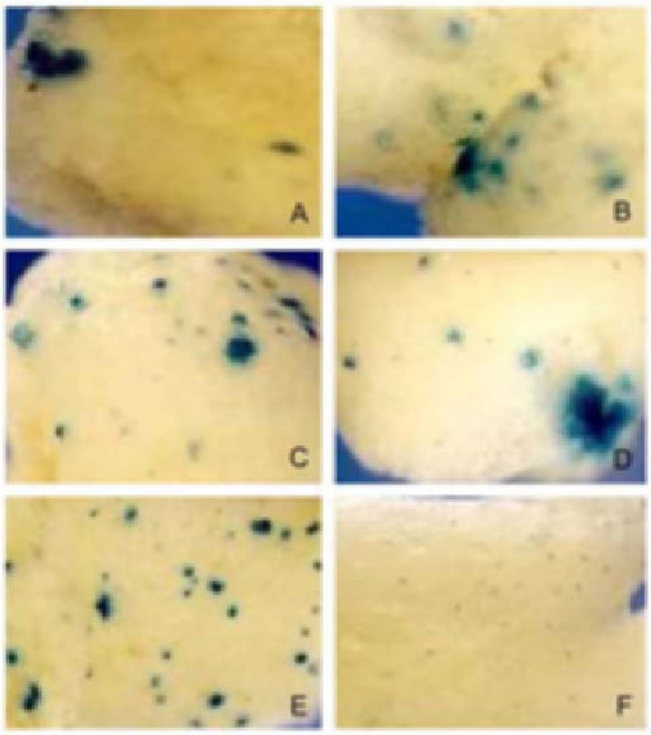

Fig. 1. Comparison of affecting parameters for bombardment in orchid protocorm; target distance of 60 mm *(A)*, target distance of 90 mm *(B)*, gold particle *(C)*, tungsten particle *(D)*, *Biolistic PDS-1000/He (E)*, and modified particle inflow gun *(F)*.

No effect of helium gas pressure on GUS activity was observed. The difference in the number of blue spots from helium gas pressure of 6 200 and 7 500 kPa was not significant (Table 1). Yang *et al.* (1999) and Nan and Kuehnle (1995) also found no difference in transient GUS expression after bombardment at various pressures of helium gas in *Dendrobium* orchid transformant. Nevertheless, with a better survival transformation rate for bombardment at 7 500 kPa, this pressure was recommended for orchid bombardment.

Two types of particle gun; *Biolistic PDS-1000/He* and modified helium particle inflow gun, *PIG* (*CAMBIA*, Canberra, Australia) were also compared for their efficiency in genetic transformation. The result showed that the blue spots on the protocorm bombarded with the *Biolistic PDS-1000/He* (Fig. 1*E*) were more intense than those using the *PIG* gun (Fig. 1*F*). The *Biolistic PDS-1000/He* was not only superior in controlling bombardment parameters, but also helpful in decreasing contamination (Nan and Kuehnle 1995) and safer than the modified helium particle inflow gun (Klein *et al.* 1987).

Table 1. *Gus* gene expression and survival percentage of orchid protocorms 3 d after bombardment with different parameters.

Parameters		Number of GUS spots [protocorm^{-1}]	Survival [%]
Type of particle	gold	28.16a	65.77
	tungsten	23.44a	57.14
He gas pressure	6200 kPa	25.26b	48.89
	7500 kPa	26.34b	73.99
Protocorm size	1 - 2 mm	26.95c	61.45
	3 - 5 mm	24.65c	100.00

In previous reports, orchid protocorms were widely used for genetic transformation to recover transgenic orchids such as *Dendrobium* (Kuehnle and Sugii 1992, Chia *et al.* 1994, Yu *et al.* 1999, 2001, Men *et al.* 2003), *Phalaenopsis* (Anzai *et al.* 1996, Chai *et al.* 2002, Liao *et al.* 2004), *Cymbidium* (Yang *et al.* 1999), *Brassia, Cattleya, Doritaenopsis* (Knapp *et al.* 2000), and *Oncidium* (Liau *et al.* 2003, You *et al.* 2003). Nan and Kuehnle (1995) showed that the protocorm-like body of *Dendrobium* orchid was the target tissue that yielded the highest GUS expression. The sizes of the protocorms, however, also affected their survival percentage and transient GUS expression. Table 1 show that the survivals of small and large protocorm were 61.45 and 100 %, respectively. It was demonstrated that bombardment could damage small protocorms, but did not affect the large protocorms. However, transgene integration is favoured in cells which are in the M-and G_2-phases to cells in the S-and G_1-phases (Iida *et al.* 1991), and the small protocorms with most of their cells in the dividing state gave a higher number of blue spots and more efficient transformation than the large protocorms with most of their cells in stationary state (Table 1). The results suggested that the target tissue must be actively dividing cells, which can be obtained by sub-culturing

them in liquid medium to stimulate cell division prior bombardment to accomplish orchid transformation.

Selection and GFP expression in transgenic orchids: The *Biolistic PDS-1000/He* accelerating gold particles with a helium gas pressure of 7 500 kPa was utilized for bombardment of protocorms with pMNK1005 plasmid carrying the *hph* and *gfp* genes driven by the Ubi-1 promoter and Nos terminator. Various stages of GFP expression of bombarded protocorms were observed during the time period of hygromycin selection. Our previous study showed that solid MS medium supplemented with 30 mg dm^{-3} hygromycin was able to completely inhibit the growth of all *Dendrobium* tested within two months; consequently, it was used for the selection of transgenic orchids in this experiment.

The concentration and timing for the selection process are also important factors that determine the efficiency of transformation (Tee *et al.* 2003). Normally, orchid protocorms for transformation are slowly proliferating and might be chimera because of de novo embryogenesis, which directly regenerate into plantlets without passing through the callus, especially in *Dendrobium* (Kuehnle and Sugii 1992). Besides, Yang *et al.* (1999) demonstrated that selection in solid medium with a high concentration of selective agent immediately after bombardment could not obtain transgenic orchids, while delayed selection would be of very low efficiency (Yu *et al.* 1999, Men *et al.* 2003) and would often obtain chimeras (Christou and Ford 1995). Hence, in this experiment, selection was divided into three steps.

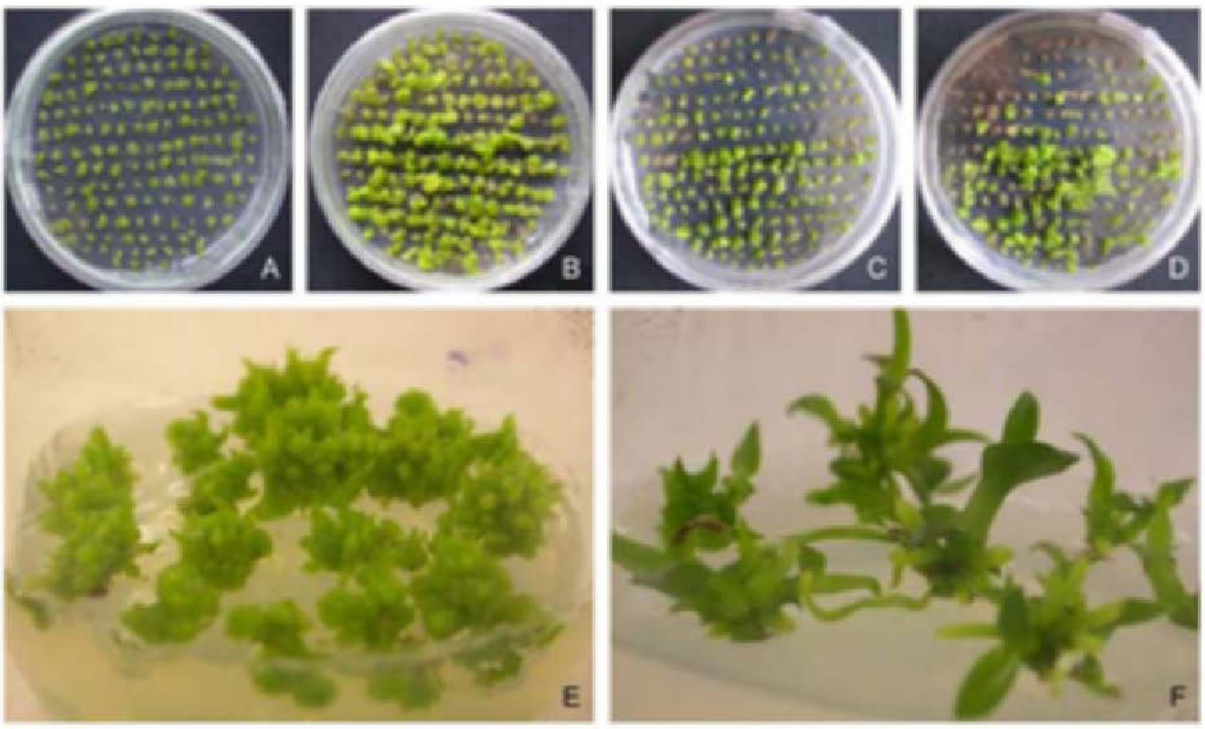

Fig. 2. Characters of tissues during hygromycin selection; 3 d *(A)*, 45 d *(B)*, 90 d *(C)*, 120 d *(D)*, hygromycin-resistance plb *(E)*, and putative transgenic plantlets *(F)*.

For 45 d of the first selection, the protocorms were weakly selected from solid MS medium supplemented with 5 mg dm^{-3} hygromycin (Fig. 2*A*). The protocorms after bombardment showed small green fluorescent spots of transformed cells and red-orange backgrounds of un-transformed cells (Fig. 3*A*). Within 30 d of the first selection, 15.33 % of all bombarded protocorms turned brown and died before the remaining green tissue further proliferated rapidly (Fig. 2*B*). The spots of green fluorescent were bigger, whereas almost all of the tissues would consist of un-bombarded cells (Fig. 3*B*). It was implied that the medium containing 5 mg dm^{-3} hygromycin could maintain and allow proliferation of the transformed tissues. All surviving protocorms were subsequently transferred into solid MS medium containing 25 mg dm^{-3} hygromycin for 45 d. Although, in this selection, approximately half of the protocorms deteriorated and died (Fig. 2*C*), the green fluorescent spots were larger (Fig. 3*C*). At this selection stage, the percentage of living protocorms was 50.96 %. Yang *et al.* (1999) suggested that to obtain transformed cells, DNA has to be integrated into the plant genome at an early stage which immediately stimulates cell division during antibiotic selection. Hence, stepwise selection in hygromycin at 5 and 25 mg dm^{-3} was beneficial in eliminating the un-transformed cells and simultaneously

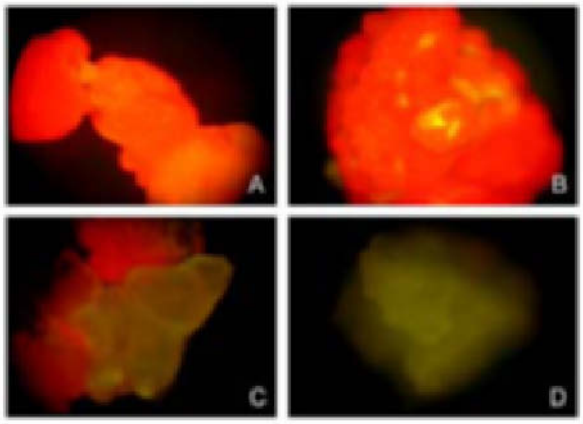

Fig. 3. GFP expressing cells after hygromycin selection; first week selection *(A)*, 45 d *(B)*, 90 d *(C)*, and 120 d *(D)*.

promoting cell division in transformed tissues.

Surviving tissues in 25 mg dm^{-3} hygromycin were transferred into solid MS medium containing 30 mg dm^{-3} hygromycin for 30 d. The transformed tissues were still green and subsequently proliferated, while untransformed tissues were completely dead after the selection (Fig. 2*D*). Hygromycin-resistant protocorms resulting from the selection strategy contained *gfp*-transformed cells that could express GFP and did not obtain non GFP-expressing cells (Fig. 3*D*). The transformation efficiency of 19.87 % was rather high when compared with the former reports (Nan and Kuehnle 1995, Anzai *et al.* 1996, Yang *et al.* 1999, Yu *et al.* 1999, Knapp 2000, Men *et al.* 2003, You *et al.* 2003). After the selection process, the hygromycin-resistant tissues which initially formed shoots were transferred into solid MS medium without antibiotic (Fig. 2*E*) to produce putative transgenic plantlets (Fig. 2*F*). However, the level of hygromycin as a selective agent and timing for selection in *Dendrobium* orchids also greatly depend on species. Yu *et al.* (1999) reported that hygromycin at 50 mg dm^{-3} for 60 d completely suppressed the growth of untransformed *Dendrobium hybrid* 'MiHua' protocorms and transgenic *Dendrobium, Phalaenopsis* and *Dendrobium nobile* plantlets were obtained by selecting from 30 mg dm^{-3} hygromycin for 105 d (Men *et al.* 2003), while in our experiment, the concentration of hygromycin needed to eliminate untransformed cells from transformed cells of *Dendrobium* Jaquelyn Thomas protocorm was 30 mg dm^{-3} for 60 d.

Using too high concentration of antibiotic to discriminate untransformed orchid tissue might result in chimeras due to escape (Chia *et al.* 1994), or death of transformed tissue. The problem of chimeras has also been reported in transgenic soybean (Christou *et al.* 1989) and papaya (Fitch *et al.* 1990). Moreover, long-term selection with low level of antibiotic would inhibit the regeneration of transgenic orchid (Kuehnle and Sugii 1992) and not completely suppress the growth of untransformed tissues (Griesbash 1994, Nan and Kuehnle 1995). The inhibitory activity of antibiotics on regeneration of transgenic plantlets has also been reported in *Nicotiana* (Kiernan *et al.* 1989) and *Oryza* (Dekeyser *et al.* 1989). In basmati rice transformation, hygromycin at 30 mg dm^{-3} was optimal for effective selection, whereas, higher concentrations, *i.e.*, 40 or 50 mg dm^{-3} were toxic. Although there were no escape of untransformed cells, regeneration efficiency of the transformed tissue was rather low (Grewal *et al.* 2006). To solve these problems, a stepwise selection was adapted using solid MS medium containing hygromycin at 5 mg dm^{-3} and 25 mg dm^{-3} each for 45 d prior selection in 30 mg dm^{-3} for 30 d to avoid the occurrence of chimeras or the inhibition of plantlet formation. The results demonstrated the accomplishment of this selection strategy to recover non-chimera orchid transformants with high efficiency.

PCR and Southern blot analysis of transgenic plant: PCR and southern blot analysis were used for detection of the integrated transgene in the genome of putative transgenic orchids. PCR analysis showed the presence of exogenous *hph* (Fig. 4*A*) and *gfp* (Fig. 4*C*) in the positive control and all eight putatively transgenic orchids, 6, 12, 34, 35, 36, 37, 39, 40, with two different sets of *hph* and *gfp* primers. There was no amplification of DNA from negative control. Southern blot analysis of the PCR products presenting *hph* (Fig. 4*B*) and *gfp* (Fig. 4*D*) showed 0.5 and 0.7 kp bands which hybridized with *hph* and *gfp* probes, respectively. All eight independent putative transgenic lines contained both 0.5 kb *hph* and 0.7 kb *gfp* gene fragments. Neither the *hph* nor the *gfp* hybridization signal was found in the untransformed control line.

To confirm *gfp* integration in the genome of the putative transgenic plantlets, 10 μg of genomic DNA from leaves of un-bombarded orchid and eight transgenic lines were digested with *Sac*I and hybridized with a 0.7-kb of *gfp* fragment probe. As this plasmid carries a unique *Sac*I site, digestion of the genomic DNA of

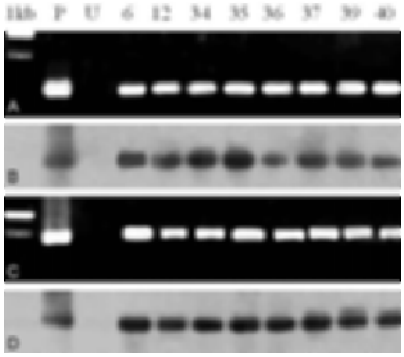

Fig. 4. PCR and Southern blot hybridization of *hph* and *gfp*; the 0.5-kb of *hph* fragment of PCR analysis *(A)*, the 0.5-kb of *hph* fragment of PCR southern blot hybridization *(B)*, the 0.7-kb of *gfp* fragment of PCR analysis *(C)*, and the 0.7-kb of *gfp* fragment of PCR Southern blot hybridization *(D)*. 1 kb-marker, positive (P) control, un-bombarded (U) line and independent putative transgenic lines (6, 12, 34, 35, 36, 37, 39 and 40).

putative transformants with *Sac*I would generate a different fragment for each integrated copy. Southern blot hybridization, therefore, provided copy numbers of the *gfp* in the genome of the putative transgenic plantlets. Unique *gfp* specific banding patterns, indicating independent transformation events, occurred in all putatively transgenic lines (Fig. 5). The hybridization revealed one of the transgenic lines, 39, carried one copy, while 36 and 37 carried two copies, 12 three copies, 6 and 35 four copies, and 34 and 40 five copies, indicating multiple integrations in the genome of the transgenic orchids. A previous result for bombardment transformation in *Dendrobium* orchid also showed multiple copies of gene (Yu *et al.* 2001). A 0.7 kb band of pMNK1005 plasmid digested with *Nco*I was detected, whereas no signal of genomic DNA from leaf of unbombarded orchid was obtained. The results demonstrated that this strategy could deliver transgene into the genome producing stable transgenic lines.

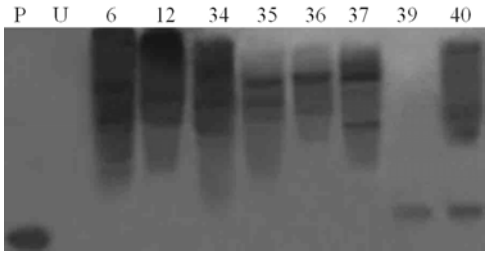

Fig. 5. Genomic Southern blot hybridization; 0.1 μg of positive (*P*) control, and 10 μg of genomic DNA digested with *Sac*I from leaves of un-transformed (*U*) plant and eight transgenic lines (6, 12, 34, 35, 36, 37, 39 and 40) were hybridized with *gfp* fragment probe.

In conclusion, this work provided the optimal conditions for bombardment transformation in delivering gene into *Dendrobium* protocorms. To solve the problems of slow growth rate and occurrence of chimera, a selection strategy was developed to obtain the highly efficient transformation (over 19%) with non-chimera tissue by selection in hygromycin at 5 and 25 mg dm^{-3} before a stringent selection regime at 30 mg dm^{-3} for complete elimination of untransformed cells. GFP detection was utilized to trace the GFP-expressing cells during selection, which facilitated observation of the transformed protocorms during selection. We believe that using the transformation system for *Dendrobium* presented here will be a powerful tool for orchid improvement.

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