

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

C. SUWANAKETCHANATIT, J. PILUEK, S. PIYACHOKANAKUL, P. S. HUEHNE*

C. SUWANAKETCHANATIT, S. PIYACHOKANAKUL
Department of Genetics, Faculty of Science, Kasetsart University, Jatuchak, Bangkok, 10900, Thailand
Tel.: +66-2-9428716, Fax: +66-2-5795528

J. PILUEK
Department of Horticulture, Faculty of Agriculture, Kasetsart University, Jatuchak, Bangkok, 10900, Thailand
Tel.: +66-2-5790308, Fax: +66-2-5795528

P. S. HUEHNE*
Department of Genetics, Faculty of Science, Kasetsart University, Jatuchak, Bangkok, 10900, Thailand
Tel.: +66-2-9428716, Fax: +66-2-5795528
Biotechnology Laboratory, Chulabhorn Research Institute, Laksi, Bangkok 10210, Thailand
Tel.: +66-2-5740522-33, Fax: +66-2-5740618

*Corresponding author:

P. S. Huehne

Department of Genetics, Faculty of Science, Kasetsart University, Jatuchak, Bangkok, 10900, Thailand, Tel.: +66-2-9428716 ext 121, Fax: +66-2-5795528
e-mail: fscipns@ku.ac.th

Abstract

Several parameters affecting transient GUS expression in protocorms of *Dendrobium* Jaquelyn Thomas were intensively examined using bombardment technique with pActin-1D plasmid. The GUS activity determined by number of blue spots on *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 showed non-chimera cells. 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: *Dendrobium* orchid; Bombardment; Transformation; Green fluorescent protein

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 colors, 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 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 microprojectile 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 ($450\text{--}600\text{ mg dm}^{-3}$) or kanamycin ($500\text{--}600\text{ mg dm}^{-3}$) 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 ($50\text{--}100\text{ mg dm}^{-3}$) 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.* 2002, Chai *et al.* 2002, Liao *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

Plant materials and culture conditions: A green capsule of *Dendrobium* Jaquelyne Thomas obtained from Rapee Sagarik Orchid Garden at Kasetsart, Bangkok, University, Thailand was immersed in 70% ethanol, which was ignited for surface-sterilization, 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 cm³ 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 cm³ of liquid ½ MS (Murashige and Skoog 1962) medium containing 15 g dm⁻³ sucrose and 75 cm³ 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.

Plasmid: 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 manitol, 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, California). 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 1mM X-gluc, 100mM sodium phosphate buffer, pH 8.0, 0.5mM potassium ferricyanide, and 0.5mM 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, Japan) equipped with a BZA filter (Nikon, Tokyo, Japan), 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 labeling (Amersham Bioscience, 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 *SacI* were separated in a 1% of agarose gel and blotted onto Hybond N⁺ nylon membrane (Amersham Bioscience, UK) using capillary blotting with 10X SSC. The blotted membrane was hybridized at 60 °C with the *gfp* probe. After hybridization, the membrane was washed twice with 1st washing buffer (2M urea, 1 g dm⁻³ SDS, 50mM NaH₂PO₄, 150mM NaCl, 1mM MgCl₂, and 2 g dm⁻³ blocking reagent) for 10 min at 60 °C, and then re-washed with 2nd buffer (1mM Tris base, 2M NaCl, and 2mM MgCl₂) twice at room temperature. The signal was detected using CDP star substrate (Amersham Bioscience, UK) and exposed the signal on X-ray film (Kodak, USA) 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, levels of helium gas pressure, 6,200 and 7,500 kPa, 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 90mm (Fig. 1B) were more spreader-out than 60 mm (Fig. 1A). The target distance of 90 mm, therefore, was a suitable parameter to be used for further orchid transformation.

Table1 *gus* gene expression and survival percentage of orchid protocorms three days after bombardment with different parameters

Parameters	Average number of GUS spots per protocorm	Survival percentage
Type of particles		
Gold	28.16a	65.77
Tungsten	23.44a	57.14
He gas pressure (psi)		
900	25.26b	48.89
1,100	26.34b	73.99
Size of target protocorm		
Small	26.95 ^D c	61.45
Large	24.65c	100.00

D: comparison with equal surface area of protocorm

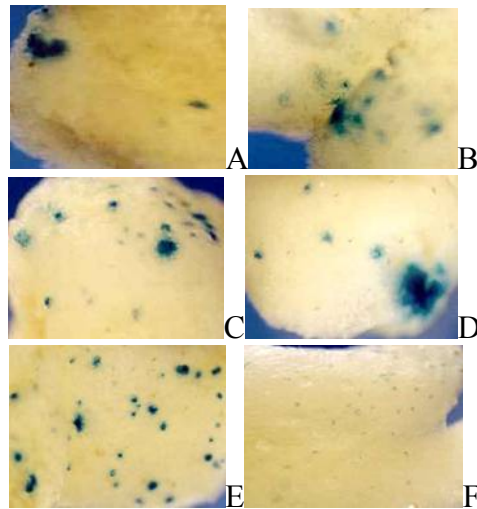


Fig.1A-F Comparison of affecting parameters for bombardment in orchid protocorm. A target distance of 60mm. B target distance of 90mm. C gold particle. D tungsten particle. E Biolistic PDH1000/He F modified particle inflow gun

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. 1C), while the irregular surfaced of sharp tungsten particles gave a large blue patch (Fig. 1D) 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.

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. 1E) were more intense than those using the PIG gun (Fig. 1F). 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). Thus, the Biolistic PDS-1000/He was considered to be an efficient gun for establishing transformants.

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, Yu *et al.* 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. Although, because of the fact that transgene integration is favored in cells which are in the M-and G₂-phases to cells in the S-and G₁-phases (Iida *et al.* 1991), 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 pMNG1005 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.

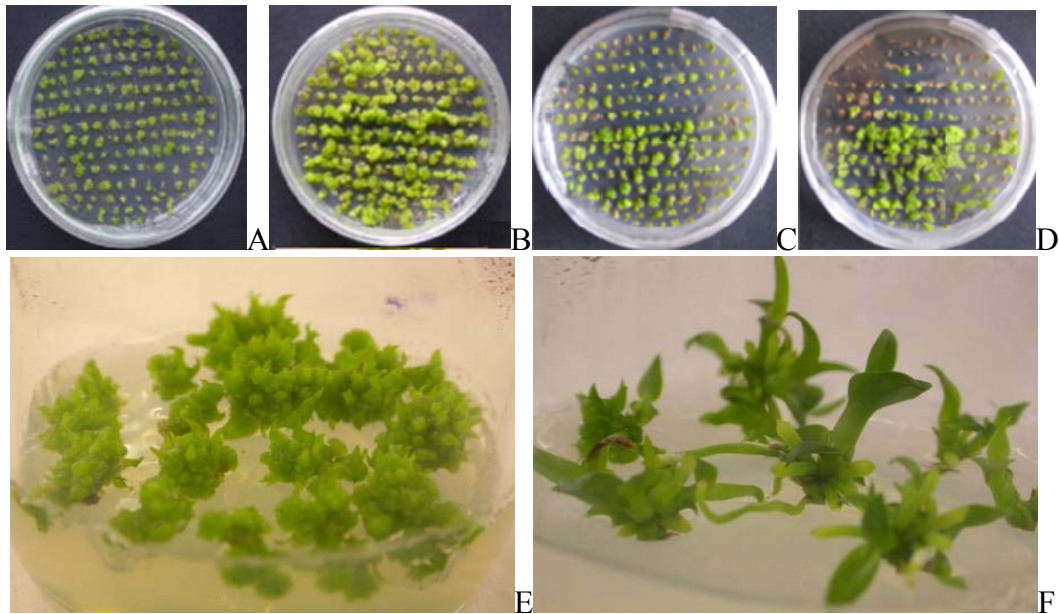


Fig.2A-F Characters of tissues during hygromycin selection. A 3 days. B 45 days. C 90 days. D 120 days E hygromycin-resistance plbs. F putative transgenic plantlets

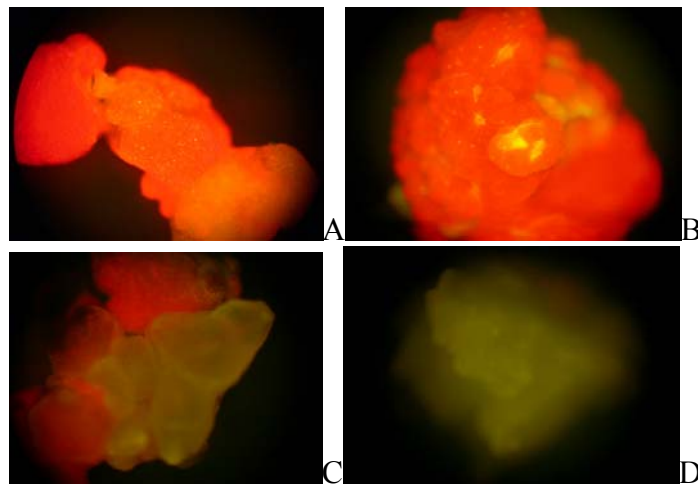


Fig.3A-D GFP expressing cells after hygromycin selection. A first week selection B 45 days C 90 days D 120 days

For 45 d of the first selection, the protocorms were weakly selected from solid MS medium supplemented with 5 mg dm^{-3} hygromycin (Fig. 2A). The protocorms after bombardment showed small green fluorescent spots of transformed cells and red-orange backgrounds of un-transformed cells (Fig. 3A). 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. 2B). The spots of green fluorescent were bigger, whereas almost all of the tissues would consist of un-bombarded cells (Fig. 3B). 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. 2C), the green fluorescent spots were larger (Fig. 3C). 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 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. Fig. 2D shows that transformed tissues were still green and subsequently proliferated, while untransformed tissues were completely dead after the selection. 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. 3D). 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. 2E) to produce putative transgenic plantlets (Fig. 2F). 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 Jaquelyne Thomas* protocorm was 30 mg dm⁻³ for 60 d.

Using too high a level 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). 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. 4A) and *gfp* (Fig. 4C) in the positive control (lane 2) and all eight putatively transgenic

orchids, 6, 12, 34, 35, 36, 37, 39, 40, (lane 4-11) with two different sets of *hph* and *gfp* primers. There was no amplification of DNA from negative control (lane 3). Southern blot analysis of the PCR products presenting *hph* (Fig. 4B) and *gfp* (Fig. 4D) showed 0.5-kb 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 (lanes 4-11). Neither the *hph* nor the *gfp* hybridization signal was found in the untransformed control line (lane 3).

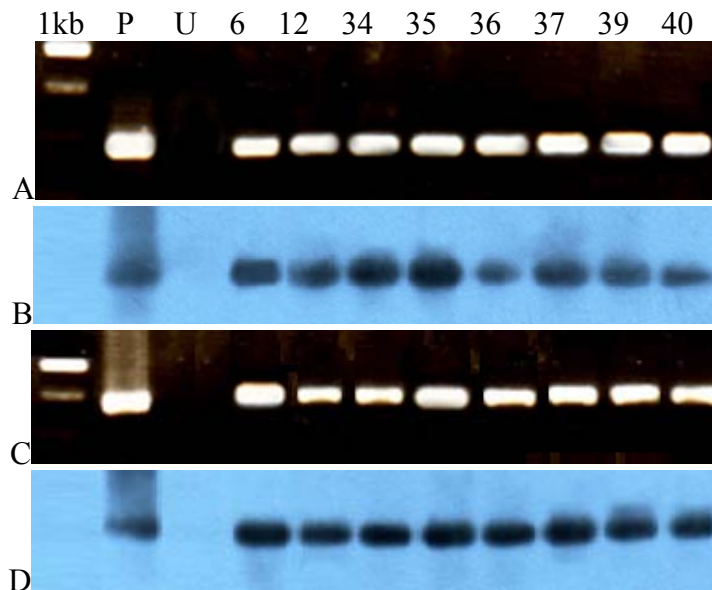


Fig.4A-D PCR and PCR Southern blot hybridization of *hpt* and *gfp*. A, B the 0.5-kb of *hpt* fragment of PCR analysis (A) of PCR Southern blot hybridization (B). C, D the 0.7-kb of *gfp* fragment of PCR analysis (C) and of PCR Southern blot hybridization (D). 1kb-marker (lane1), positive (P) control (lane 2), un-bombarded (U) line (lane 3) and independent putative transgenic lines (lane 4-11)

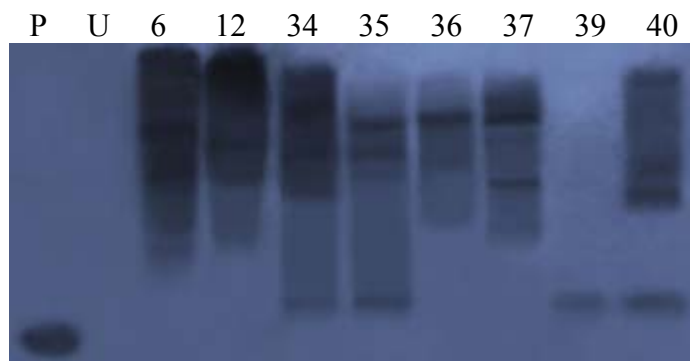


Fig.5 Genomic Southern blot hybridization. 0.1µg of plasmid DNA (lane1) 10µg of genomic DNA digested with *SacI* from leaves of untransformed plant (lane2) eight transgenic plantlets (lane 3-10) were hybridized with *gfp* fragment probe

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 *SacI* and hybridized with a 0.7-kb of *gfp* fragment probe. As

this plasmid carries a unique *SacI* site, digestion of the genomic DNA of putative transformants with *SacI* 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 (lane 9), while 36 (lane 7) lines and 37 (lane 8) carried two copies, 12 (lane 4), three copies, 6 (lane 3) and 35 (lane 6), four copies, and 34 (lane 5) and 40 (lane 10), 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 *NcoI* was detected (lane 1), whereas no signal of genomic DNA from leaf of unbombarded orchid was obtained (lane 2). The results demonstrated that this strategy could deliver transgene into the genome producing stable transgenic lines. 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 mg dm⁻³ 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.

Acknowledgments

We are grateful to Assi. Prof. Dr. James R. Ketudat-Cairns (Suranree University of Technology, Thailand) and Dr. Paiboon Vattanaviboon (Chulabhorn Research Institute, Thailand) for their help in preparing the manuscript, Prof. Dr. R. Wu (Cornell University, Ithaca, NY) for the gift of the pActin1-D plasmid, and Dr. N.M. Upadhyaya (Plant Industry, CSIRO, Australia) for providing the pMNK1005 plasmid. This work was supported by grants from the Thailand Research Fund and National Center for Genetic Engineering and Biotechnology, Thailand.

References

- Anzai, H., Ishii, Y., Shichinohe, M., Katsumata, K., Nojiri, C., Tanaka, M.: Transformation of *Phalaenopsis* by Particle Bombardment. - Plant Tissue Cult. Lett. 13: 265-272, 1996.
- Belarmino, M.M., Mii, M.: *Agrobacterium*-mediated genetic transformation of a *phalaenopsis* orchid. - Plant Cell Rep. 19: 435-442, 2000.
- Chai, M.L., Xu, C.J., Senthil, K.K., Kim, J.Y., Kim, D.H.: Stable transformation of protocorm-like bodies in *Phalaenopsis* orchid mediated by *Agrobacterium tumefaciens*. - Scientia Hort. 96: 213-224, 2002.

- Chia, T.F., Chan, Y.S., Chua N.H.: The firefly luciferase gene as a non-invasive reporter for *Dendrobium* transformation. - Plant J. 6: 441-446, 1994.
- Christou, P., Ford, T.L.: The impact of selection parameters on the phenotype and genotype of transgenic rice callus and plants. - Transgenic Res. 4: 44-51, 1995.
- Christou, P., Ford, T.L., Kofron, M.: Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/ Technol. 9: 957-962, 1991.
- Christou, P., Swain, W., Yang, N.S., McCabe, D.: Inheritance and expression of foreign genes in transgenic soybean plants. - Proc. Natl. Acad. Sci. USA. 86: 7500-7504, 1989.
- Dekeyser, R., Claes, B., Marichal, M., Montagu, M.V., Caplan, A.: Evaluation of selectable markers for rice transformation. - Plant Physiol. 90: 217-223, 1989.
- Fitch, M., Manshardt, R., Gonsalves, D., Slightom, J., Sanford, J.: Stable transformation of papaya via microprojectile bombardment. - Plant Cell Rep. 9: 189-194, 1990.
- Griesbach, R.J.: An improved method for transforming plants through electrophoresis. - Plant Sci. 102: 81-89, 1994.
- Hunold, R., Bronner, R., Hahne, G.: Early events in microprojectile bombardment: cell viability and particle location. - Plant J. 5: 593-604, 1994.
- Iida, A., Yamashida, T., Yamada, Y., Morikawa, H.: Efficiency of particle-bombardment-mediated transformation is influenced by cell stage in synchronized cultured cells of tobacco. - Plant Physiol. 97: 1585-1587, 1991.
- Jefferson, R.A., Kavanagh, T.A., Beman, M.W.: Gus Fusion: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. - EMBO J. 6: 3901-3907, 1987.
- Kiernan, J.M., Goldberg, K.B., Young, M.J., Schoelz, J.E., Shepherd, R.J.: Transformation and regeneration of *Nicotiana edwardsonii*. - Plant Sci. 64: 67-78, 1989.
- Klein, T.M., Wolf, E.D., Wu, R., Sanford, J.C.: High velocity microprojectiles for delivering nucleic acids into living cells. - Nature. 327: 70-73, 1987.
- Knapp, J.E., Kausch, A.P., Chandlee, J.M.: Transformation of three genera of orchid using the *bar* gene as a selectable marker. - Plant Cell Rep. 19: 893-898, 2000.
- Kuehnle, A.R., Sugii, N.: Transformation of *Dendrobium* orchid using particle bombardment of protocorm. - Plant Cell Rep. 11: 484-488, 1992.
- Liao, L.J., Pan, I.C., Chan, Y.L., Hsu, U.H., Chen, W.H., Chan, M.T.: Transgene silencing in *Phalenopsis* expressing the coat protein of Cymbidium Mosaic Virus is a manifestation of RNA-mediated resistance. - Mol. Breeding. 13: 229-242, 2004.
- Liau, C.H., You, S.J., Prasad, V., Hsiao, H.H., Lu, J.C., Yang, N.S., Chan, M.T.: *Agrobacterium tumefaciens*-mediated transformation of an *Oncidium* orchid. - Plant Cell Rep. 21: 993-998, 2003.
- Men, S., Ming, X., Wang, Y., Liu, R., Wei, C., Li, Y.: Genetic transformation of two species of orchid by biolistic bombardment. - Plant Cell Rep. 21: 592-598, 2003.
- Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiologia Pl. 15: 473-497, 1962.

- Murray, M.G., Thompson, W.F.: Rapid isolation of high molecular weight plant DNA. - *Nucleic Acid Res.* 8: 4321–4325, 1980.
- Nan, G.L., Kuehnle, A.R.: Factor effecting gene delivery by particle bombardment of *Dendrobium* orchid. - *In Vitro Cell Dev. Biol. Plant.* 31: 131-136, 1995.
- Russell, J.A., Roy, M.K., Sanford, J.C.: Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. - *Plant Physiol.* 98: 1050-1056, 1992.
- Sanford, J.V., Smith, F.D., Russell, J.A.: Optimizing the biolistic process for different biological applications. - *Methods Enzymol.* 217: 483-509, 1993.
- Tee, C.S., Marziah, M., Tan, C.S., Abdullah, N.P.: 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, 2003.
- Upadhyaya, N.M., Ramm, K., Gaudron, J., Craig, S., Wang, M.B., Gupta, S., Okita, T.W., Waterhouse, P.M.: Can *gfp* replace *uidA* as a reporter gene to monitor transformation of cereals by biolistics or *Agrobacterium*? - In: Larkin, P.J. (ed.): *Proceedings of the 4th Asia-Pacific Conference on Agricultural Biotechnology*. Pp. 111-113. CPN Publications, Fyshwick - Canberra 1998.
- Vacin, E., Went, F.: Some pH changes in nutrients solutions. - *Bot. Gaz.* 110: 605-613, 1949.
- Yang, J., Lee, H.J., Shin, D.H., Ok, S.K., Seon, J.H., Paek, K.Y., Han, K.H.: Genetic transformation of *Cymbidium* orchid by particle bombardment. - *Plant Cell Rep.* 18: 978-984, 1999.
- You, S.J., Liao, C.H., Huang, H.E., Feng, T.Y., Prasad, V., Hsiao, H.H., Lu, J.C., Chan, M.T.: Sweet pepper ferredoxin-like protein (*pflp*) gene as a novel selection marker for orchid transformation. - *Planta.* 217: 60-65, 2003.
- Yu, Z., Chen, M., Nie, L., Lu, H., Ming, X., Zheng, H., Qu, L.J., Chen, Z.: Recovery of transgenic orchid plants with hygromycin selection by particle bombardment to protocorms. - *Plant Cell Tissue Organ Cult.* 58: 87-92, 1999.
- Yu, H., Yang, S.H., Goh, C.J.: *Agrobacterium*- mediated transformation of a *Dendrobium* orchid with the class 1 *knox* gene DOH1. - *Plant Cell Rep.* 20: 301-305, 2001.