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Genetic transformation of *Vanda lilacina* Teijsm. & Binnend. with a chitinase gene

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Abstract. This research was aimed to improve plantlet regeneration and to establish the optimal conditions for transformation of *Vanda lilacina* Teijsm. and Binnend. using *Agrobacterium tumefaciens* strain LBA4404 (pCAMBIA 1305.1) harboring a chitinase gene as a fungal resistant one. It was found that seeds successfully germinated, and developed into protocorms and plantlets on solid New Dogashima (ND) medium supplemented with 1 % (w/v) potato juice. Protocorm proliferation was successful when culturing the protocorms on solid ND medium supplemented with 3 mg/l BA. For successful genetic modification, the protocorms were cocultivated with *A. tumefaciens* for 45 min. Bacterial elimination and selection of putative transformants were implemented using 250 mg/l cefotaxime and 10 mg/l hygromycin, respectively. GUS assay revealed 80% of the protocorms expressing *gus* gene activity. Availability of 35S and NOS detected by PCR analysis confirmed gene integration.
Key Words: *Vanda lilacina*, *Agrobacterium tumefaciens*, chitinase, cefotaxime, hygromycin.

Introduction. Orchidaceae is the largest family of the flowering plants. It consists of 779 genera and 22,500 species (Mabberley 2008). About 177 genera and 1,135 species are found in Thailand (Atichart et al 2007). The species *Vanda lilacina* Teijsm. & Binnend., native to north and northeast Thailand, is one of the orchids which have been widely propagated as ornamental plants and as cut flowers traded within the country and exported to some foreign countries. In general, the orchid growers and orchid industry employ classical breeding methods via sexual hybridization and selection to create novel and improved orchids. The classical breeding methods are considered disadvantageous as they are time-consuming and they are unable to produce disease- and pest-free orchids. Pest and disease is considered crucial involving growth and development hindrance. This is a main problem in orchid production. With the advent of genetic engineering technology, relatively rapid propagation and selection of genetically-modified orchids with desirable or novel traits have now become feasible (Chai & Hu 2007). Gene transfer technology overcomes limitations of traditional breeding methods and regeneration process is less time-consuming than conventional hybridization methods (Subramaniam et al 2009). *Agrobacteriam*-mediated transformation is one of genetic transformation systems which are widely employed to create desirable traits of many plant species. It is the most effective procedure for introducing foreign DNA or transgene into plant genomic DNA. Also, it has remarkable advantages over direct transformation methods as it reduces the copy number of the transgene, potentially leading to fewer problems with transgene cosuppression and instability (Hansen et al 1994). Besides, it does not require construction of a particular vector and thus is host-independent (Janna et al 2006).

A success in shipping transgene into plant cells with successful integration into plant genome via *Agrobacterium* relies on multifactors. Optimal conditions for each species, therefore, should be studied. Here, we provided optimal conditions for *Agrobacterium*-mediated transformation of *V. lilacina* with a chitinase gene in an attempt to create fungal-free orchids.

Material and Method. Seed-derived protocorms were used as explants in the study. Three-month-old capsules of *V. lilacina* were gently washed with detergent and subsequently rinsed with running tap water for 1 h prior to surface sterilization. They were then dipped into 70% (v/v) ethanol for 5 min and subsequently soaked in a disinfectant, 25% (v/v) sodium hypochlorite in combination with Tween-20, with continuous shake for approximately 20 min. Thereafter, they were washed for 3 times with sterile distilled water. Sterilization was repeated by dipping the capsules into 70% ethanol and immediately flaming for 3 times. After that, they were longitudinally dissected and seeds were picked off and transferred onto solid New Dogashima (ND) medium supplemented with 0-5 mg/l Benzylaminopurine (BA) in combination with 0-1 mg/l Naphthalene Acetic Acid (NAA). The cultures were kept at 25 ± 2 °C under a long photoperiod (16 h light: 8 h dark) with a photon dose of 40 µmol $m^{-2}s^{-1}$. Five replicates were conducted for each treatment. Modification of *V. lilacina* performed in the study included the steps of selecting antibiotic doses for bacterial elimination and selection of putative transformants, and determining optimal cocultivation time regarding high level of gus gene expression verified by GUS assay and successful gene integration detected by PCR technique as following.

Determination of antibiotic doses

The protocorms were cultured on solid ND medium supplemented with cefotaxime (0-450 mg/l) or hygromycin (0-40 mg/l). The cultures were kept at 25 ± 2 °C under a long photoperiod (16 h light: 8 h dark) with a photon dose of 40 µmol $m^{-2}s^{-1}$. After 4 weeks of culture, number of viable protocorms were examined. Five replicates were conducted for each treatment.

Preparation of bacteria and determination of cocultivation time

A. tumefaciens strain LBA4404 (pCAMBIA 1305.1) was cultured overnight at 25 ± 2 °C in liquid Luria Broth medium supplemented with 50 mg/l kanamycin until reaching OD_{600}=1.0. The protocorms were cocultivated with *A. tumefaciens* for 0-75 min, then dried on sterile tissue papers and transferred to the induction medium for 3 d. The optimal cocultivation time for high effectiveness of genetic transformation was determined regarding number of the protocorms showing blue coloration and level of gene expression.

Genetic transformation

The seed-derived protocorms were cocultivated with *A. tumefaciens* for 45 min, dried on sterile tissue papers and then transferred to the induction medium for 3 d. The resulted protocorms were washed thoroughly with sterile distilled water supplemented with 250 mg/l cefotaxime. They were subsequently introduced into selection step by culturing them on the same medium supplemented with 250 mg/l cefotaxime and 10 mg/l hygromycin. The viable protocorms were then transferred to the regeneration medium supplemented with the same selective agents. After 4 weeks of culture, numbers of transgenic and escaped plants were examined.

Histochemical GUS assay

Histochemical staining for GUS activity was performed in accordance with the method of Jefferson (1987). Ten transformed protocorms were transferred into a micro tube containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) and incubated overnight at 37 °C.

Detection of gene integration by Polymerase Chain Reaction (PCR) technique

Total genomic DNA was extracted from the transformants and control by CTAB method (Doyle & Doyle 1987). The primer sequences for PCR were as follows: 35S forward sequence (F)5'- GCTCCTACAAATGCCATCA-3', reverse sequence (R)5'-GATAGTG GGATTGTGCGTCA-3' to yield a 195 bp fragment; NOS (F)5'-GAATCCTGTTGCCGGTCTTG-3', (R)5'-TTATCCTAGTTTGCGCGCTA-3' to yield a 180 bp fragment. The DNA was denatured at 94 °C for 3 min, followed by 40 cycles of amplification (20 sec at 94 °C; 40 sec at 50 °C; 60 sec at 72ºC). The final incubation at 72 °C was extended to 3 min, and the reaction material was cooled and kept at 4 °C. The PCR products were visualized by running the completed reaction on a 2% agarose gel containing ethidium bromide.

Results and Discussion. *V. lilacina* seeds successfully germinated and developed into protocorms after culture on ND medium for 4 weeks. The results showed that the protocorm cultured on solid ND medium supplemented with 3 mg/l BA (Type G) gave rise to the highest proliferation capacity of the protocorms (Table 1 & Figure 1). Generally, plant growth regulators are vital for growth promotion. They are required either singly or ordinarily in combination. Here, the results suggested that BA alone was sufficient to promote protocorm proliferation in this species. This was consistent to the the study by Kong et al (2007) reporting that addition of 0.5 mg/l 6-BA in media did not promote shoot differentiation but enhanced protocorm proliferation in *Dendrobium strongylanthum*.

Table 1

Protocorm proliferation after 4 week of culture on solid ND medium supplemented with 1% potato extract plus NAA and/or BA

Medium type	*Amount of hormones added in media*		*Protocorm weight (mg)*[Z]
	NAA (mg/l)	*BA (mg/l)*	
A	0.0	0.0	281.7±2.7[a]
B	0.5	0.0	320.5±4.2[b]
C	1.0	0.0	397.3±2.8[c]
D	0.0	1.0	285.4±5.1[a]
E	0.5	1.0	334.7±3.6[d]
F	1.0	1.0	416.0±2.3[e]
G	0.0	3.0	813.5±3.0[f]
H	0.5	3.0	689.8±5.8[g]
I	1.0	3.0	482.0±6.3[h]
J	0.0	5.0	392.7±3.3[c]
K	0.5	5.0	369.7±3.2[i]
L	1.0	5.0	338.5±2.9[d]

[Z] – Data is the mean of five replicates and means with the same alphabet (a, b, c, d, e, f, g, h or i) are not significantly different at p=0.05 using Duncan's multiple range test.

Antibiotics had a profound effect on protocorm proliferation and viability. An increase in antibiotic concentrations resulted in a decrease in viability and proliferation capacity of the protocorms (Figures 2a & 2b). A number of viable protocorms cultured in the presence of cefotaxime gradually dropped by turning brown in color, and finally died in the following 2 weeks. In this study, 250 mg/l cefotaxime was considered appropriate for bacterial elimination on account of the fact that *A. tumefaciens* was completely eliminated while maintaining viability of the protocorms and not disturbing their proliferation capacity. A number of viable protocorms cultured in the presence of hygromycin sharply decreased by turning dark brown in color. Hygromycin concentration of 10 mg/l was suitable for selection of putative transformants for the reason that nontransformants could not survive under this condition. The cause of death in the protocorms cultured in the presence of antibiotics resulted from inhibition of transcription and translation by the antibiotics (Datta et al 1999).

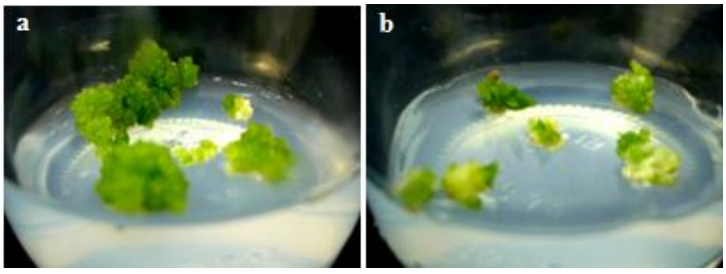

Figure 1. Protocorm proliferation in *V. lilacina*: a) Protocorms cultured on solid ND medium supplemented with 3 mg/l BA, b) Control.

In an indirect system of genetic transformation employing *A. tumefaciens* as a transgene carrier, antibiotics play an important role in both bacterial elimination and selection of transformants. Hygromycin is widely used as a selective agent in monocotyledonous transformation. In orchids, hygromycin also effectively discriminates between transformed and nontransformed cells (Belarmino & Mii 2000). A suitable concentration of antibiotics used to select transgenic plants is dependent upon plant species and some other factors. In this study, the hygromycin concentration was lower than that used to select transformants in rice cv. RD6 (Pipatpanukul et al 2004) and Phalaenopsis orchids (Mishiba et al 2005) which utilized hygromycin concentration of 20 mg/l as a selective agent.

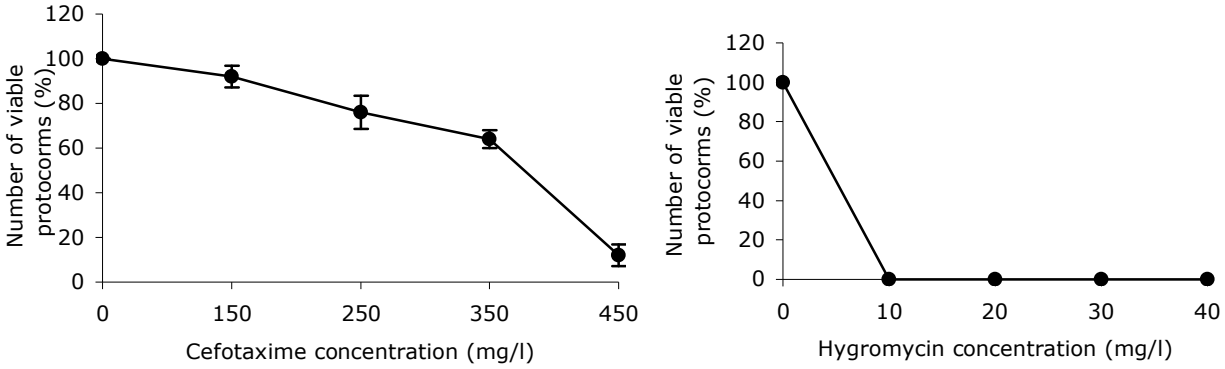

Figure 2. Antibiotic dose on viability of the protocorms after 4 weeks of culture on solid ND medium supplemented with: a) 0-450 mg/l cefotaxime, or b) 0-40 mg/l hygromycin.

A success in genetic transformation of plant species also relies on cocultivation time. The cocultivation time is considered as a crucial factor affecting the effectiveness of bacterial invasion into plant cells. The cocultivation time with *A. tumefaciens* ranges from 1-2 min to 3 d dependent upon plant species (Mendel & Hansch 1995). In this study, cocultivation time of 45 min gave rise to the highest level of *gus* gene expression of 60% (Table 2 & Figure 3). The results were inconsistent to other studies reporting different requirement in cocultivation period for high gene expression. Maneewan et al (2005) reported that 30-min cocultivation was sufficient for genetic transformation of *Oryza sativa* L. cv. Chainat1 with a chitinase gene whereas Khan et al (2003) reported that 2-3 d cocultivation was required in transformation of *Brassica napus* with *A. tumefaciens* strain EHA105. This could be inferred that cocultivation time is a limiting factor.

Table 2

Numbuer of the protocorms showing blue coloration after cocultivation with *A. tumefaciens* for 0-75 min

Cocultivation time (min)	*Number of the protocorms showing blue coloration (%)*[Z]	*Level of gus gene expression*[Y,Z]
0	0.0±0.0[a]	0±0.0[a]
30	92.0±4.3[b]	30±3.3[b]
45	81.5±2.7[c]	60±2.1[c]
60	45.2±3.2[d]	45±2.7[d]
75	0±0.0[a]	0±0.0[a]

[Y] – Level of gene expression was graded into ‚+' regarding an area of blue coloration and then reported into a percentage, [Z] - data is the mean of five replicates and means with the same alphabet (a, b, c or d) are not significantly different at p=0.05 using Duncan's multiple range test.

In accordance with the results, it was clearly seen that number of the protocorms showing blue coloration was not in accordance with level of *gus* gene expression. The

results indicated that number of the protocorms showing blue coloration dropped when cocultivation time was prolonged. The protocorms which did not show blue coloration were entirely lethal, except control. This incidence implied that excessive intrusion of *A. tumefaciens* into the protocorms because of prolonged cocultivation time led to lethal death. This was consistent to the study by Khan et al (2003) reporting that prolonged cocultivation in *Brassica napus* resulted in necrotic development in tissues and shoot regeneration were restrained owing to excessive intrusion of *A. tumefaciens* strain EHA105. Therefore, it is strongly recommended that the explants of each species be evaluated for suitable cocultivation time prior to genetic modification.

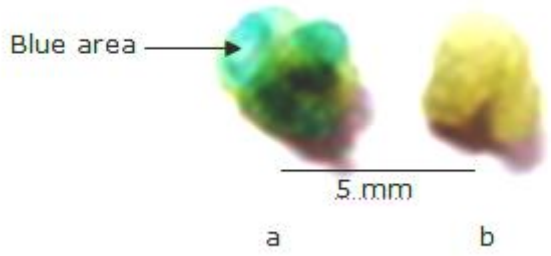

Figure 3. Blue coloration in the transformed protocorms verified by GUS assay: a) Cocultivation for 45 min, b) Control.

After a preliminary study on optimal conditions, genetic modification was conducted by which the seed-derive protocorms were cocultivated with *A. tumefaciens for* 45 min, followed by bacterial elimination using 250 mg/l cefotaxime and selection of putative transformants using 10 mg/l hygromycin. It was found that, according to histochemical GUS staining, number of the protocorms showing blue coloration was 80%. The transformed protocorms were furture cultured in the presence of selective agents in order to examine whether there were escaped plants. The results showed that there was only 5% which was considered as the escaped plants produced after 8 weeks of culture, indicating that genetic transformation via *A. tumefaciens* was quite stable.

Histochemical GUS staining is a useful tool for prescreening a success in T-DNA transfer. However, in an attempt to prove integration of transgene, PCR method is required. In the study, PCR method confirmed the integration of T-DNA by displaying the bands of 195 and 180 bp fragments in putative transformants (Figure 4). The 2 bands detected were the CaMV35S promoter and the NOS terminator, respectively. PCR amplification of the bands of 195 and 180 bp fragments was also utilized to confirm transgene integration in rice (Maneewan et al 2005) and *Dendrobium secundum* (Atichart et al 2007). The results confirmed a stable integration of transgene, thus indicating that genetic modification was truly successful.

Many factors, besides the factors cited previously, have been proposed to involve in a success in T-DNA transfer. Addition of acetosyringone, a phenolic compound released from wounded tissues in plants, has a profound influence on a success in genetic transformation in many plant species. In an indirect system of genetic modification, phenolic compounds were used to facilitate the transcriptional activation of *Agrobacterium* virulence machinery (Nadolska-Orcyk & Orczyk 2000). These compounds were transduced through a receptor *vir*A protein in the inner membrane of bacterial cells. The expression of these genes triggers transfer of T-DNA from Ti-plasmid into plant cells and integration into plant genomic DNA (Koichi et al 2002). Generally, acetosyringone was applied to a recalcitrant host; especially monocotyledonous plants e.g. orchids (Men et al 2003). There has been an application of acetosyringone in the preculturing step in genetic transformation of *Dendrobium* Savin White protocorm-like bodies and it was found that gus gene expression increased when acetosyringone concentration raised and maximum expression was found when 150 µM acetosyringone was utilized (Subramaniam et al 2009). However, gene expression decreased when acetosyringone concentration was more than 150 µM. The results suggested that high concentration of phenolic compounds may be toxic to plant cells.

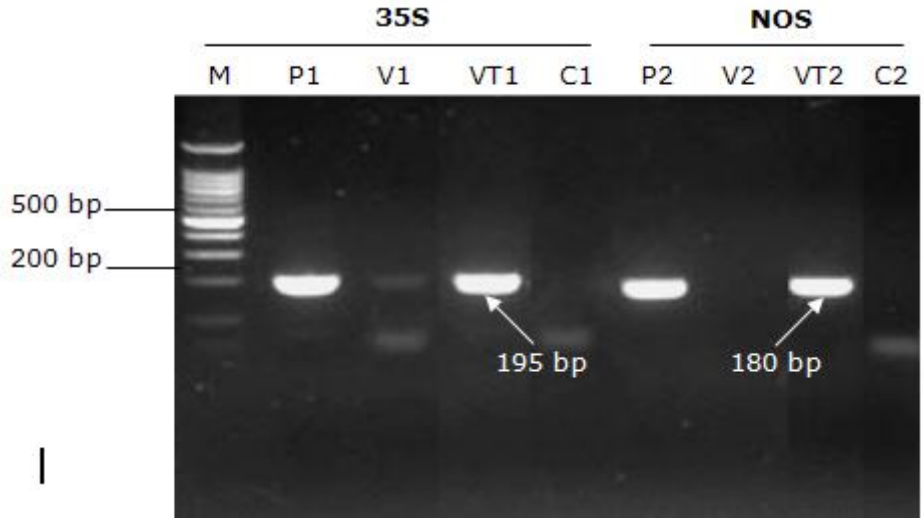

Figure 4. Detection of transgene integration by PCR technique using specific primers to detect the 35S (lane P1-C1) and NOS (lane P2-C2): M) 100 bp DNA ladder, P) pCAMBIA1305.1, V) Nontransformed protocorms, VT) Transformed protocorms, C) Control.

Calcium is another factor involving in a success in a genetic transformation system. Calcium acts as an ionic cross-linkage of the carboxyl groups of linear molecules in plant cell wall and as inducer to change the composition of cell wall which has an influence on bacterial intrusion into plant cells. In general, plant cell wall possesses 1-10 mM calcium which is more than 60% of which found in the cell (Subramaniam et al 2009). It has been reported that an increase in $CaCl_2$ could stimulate cell dissociation which was linked to a hyper secretion of polysaccharide compounds (Montoro et al 1993). Subramaniam et al (2009) reported that the highest gene expression was found in genetic transformation of *D.* Savin White when ¼-strength calcium was used while Sangwan et al (1992) reported the highest gene expression in *Arabidopsis thaliana* using calcium-free medium. These results indicated that calcium deficiency could contribute to a success in genetic modification as cell wall structure was modified and thus reduced cell wall matrix, facilitating T-DNA transfer into plant cells. In contrast, excessive calcium may be absorbed by plant cell wall and consequently strengthened the cell wall, hindering T-DNA transfer into the cells (Subramaniam et al 2009).

Here, it is recommended that all the factors cited be included in a future study in this species or other species.

Conclusions. Seed germination, protocorm proliferation and plantlet regeneration are exclusively influenced by many factors. Species, capsule age, medium type and inclusion or exclusion of plant growth regulators play a key role in a failure or success of seeds to develop. This study provided the optimal conditions for seed germination and protocorm proliferation. *V. lilacina* seeds germinated very well on solid ND medium in the absence of plant growth regulators. In an attempt to promote proliferation capacity of the protocorms, 3 mg/l BA was required according to the results. A success in modification of plants via an indirect system of genetic transformation employing *A. tumefaciens* strain LBA4404 (pCAMBIA1305.1) as a transgene carrier is dependent upon many key factors. An antibiotic concentration, tissue type and age used as explants, cocultivation time, use of phenolic compound and activeness of *A. tumefaciens* are considered crucial in this system. Here, the results suggested a successful condition for genetic transformation of *V. lilacina* as follows. The seed-derived protocorms were cocultivated with *A. tumefaciens* for 45 min, dried on sterile tissue papers and then transferred to the induction medium for 3 d. Thereafter, they were washed thoroughly with sterile distilled water supplemented with 250 mg/l cefotaxime and subsequently transferred to the selective medium supplemented with 250 mg/l cefotaxime and 10 mg/l hygromycin for 4

weeks. Subculture of transgenic plants, transient gene expression verified by GUS assay and integration of transgene detected by PCR technique were recommended to implement every month in order to exclude the escaped plants from transgenic plants.

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