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Agrobacterium-Mediated Transformation of *Dendrobium secundum* (Bl.) Lindl with Antisense ACC Oxidase

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Abstract: The suitable medium which resulted in 98% for seed germination was the Vacin and Went (1949) medium supplemented with 100 mg L^{-1} myo-inositol, 1 mg L^{-1} thiamine, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine and 4 mg L^{-1} glycine, 15% coconut water and 0.8% agar at pH 5.4 under light condition. Protocorms with high proliferation capacity were cultured on the modified VW medium and 0.5 mg L^{-1} NAA. Genetics transformation of *Dendrobium secundum* mediated by *Agrobacterium tumefaciens* strain LBA 4404, which harbored the plasmid pCAMBIA 1305.1 containing antisense ACC oxidase, β-glucuronidase (GUS) and hygromycin resistance (*hptII*) genes. The efficiency of transformation was 40 min co-cultivation of protocorms with *A. tumefaciens* that has been induced with 200 μM acetosyringone. An experiment was conducted to determine the effect of antibiotic on protocorm growth. It was found that hygromycin at 25 mg L^{-1} was effective for transformant selection. The maximum concentration of cefotaxime that protocorm could tolerate was 500 mg L^{-1}. Hygromycin resistant protocorms showed histochemical blue staining due to GUS activity was 60%. Successful the transformation was confirmed by PCR analysis. It was found that the sizes of amplified fragments were 195, 180 and 843 bp for the 35S, NOS and antisense ACC oxidase, respectively.

Key words: Protocorm, hygromycin, transformation, antisense ACC oxidase, *Agrobacterium tumefaciens*, *Dendrobium secundum*

INTRODUCTION

Orchids are the largest family of flowering plants and consist of more than 800 genera and 25,000 species, many of which are grown commercially worldwide (Arditti, 1992). Thailand has one of the richest communities of tropical orchids. It has been reported that about 177 genera and 1,135 species are found throughout the country. Dendrobium is one of the most important genera of commercial orchids used for cut flowers and potted plants. Plant genetic engineering provides an opportunity to incorporate novel traits into these orchid. The methods for plant transformation, whether it is through direct DNA delivery by Agrobacterium-mediated transformation are well established for dicotyledonous species. In recent years, the successful transformation of orchids by Agrobacterium had been reported in Phalaenopsis orchid (Belarmino and Mii, 2000; Mishiba *et al*., 2005). Agrobacterium-mediated transformation had been applied to *Dendrobium* recently. Dendrobium Madame Thong-In reported the optimization of the co-cultivation period for the transgenic orchid. Plants were regenerated by inoculating thin section explants from PLBs with *A. tumefaciens* strain LBA4404, which harbors a binary vector that carries the orchid DOH1 antisense gene. The transformation was performed through two consecutive stages of co-cultivation, with the first stage occurring on an antibiotic-free medium for 3 days and the subsequent stage occurring on a medium containing 50 mg L^{-1} carbenicillin for 3-4 weeks. Proliferated PLBs were repeatedly selected for kanamycin resistance (Yu *et al*., 2001). *A. tumefaciens* strains AGL1 and EHA105, with each containing a binary vector pCAMBIA1301 with the HPT gene as a selectable marker for hygromycin resistance and an intron-containing β-glucuronidase gene (Gus-int) as a reporter gene, were used in transformation *Dendrobium nobile* PLBs were co-cultivated with *A. tumefaciens*, which had been activated with 100 μM acetosyringone (AS), for 2-3 days until the growth of *A. tumefaciens* was observed on co-cultivation medium containing 100 μM AS following co-cultivation, PLBs were cultured on selective medium containing 30 mg L^{-1} hygromycin and 250 mg L^{-1} cefotaxime. Proliferating PLBs were repeatedly selected for hygromycin resistance. (Men *et al*., 2003).

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The postproduction quality of many flowers is limited by the increased synthesis and action of the plant hormone ethylene. Increased production of ethylene plays a role in the senescence or death of flower petals, abscission of plant parts including floral structures and discoloration of harvested (Woodson, 1994; Young *et al*., 2004). Orchid flowers are extremely sensitive to ethylene, even at low levels. Pollination and emasculation trigger ethylene production by flowers and result in rapid wilting. Blooming orchids should be handled carefully so that accidental pollination of flowers and removal of pollen caps (emasculation) do not occur (Nadeau *et al*., 1993). Ethylene is synthesized in plant tissues via the conversions of S-adenosyl-Met to 1- aminocyclopropane -1-carboxylic acid (ACC), catalyzed by ACC synthase (Kende, 1993). Inhibition of ethylene biosynthesis is possible by using antisense RNA to either ACC synthase or to ACC oxidase or by deamination of ACC (Henzi *et al*., 2000).

The objectives of this study were to improve the efficiency of regeneration and transformation of *Dendrobium secundum* with antisense ACC oxidase gene by using *Agrobacterium tumefaciens* for prolonging the longevity and vase life of orchid flowers.

MATERIALS AND METHODS

The experiments were carried out at the Gene Transfer Laboratory, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand.

Protocorm preparation and protocorm induction: Seed pods of *D. secundum* were initially washed with a mild detergent solution and then soaked in 15% (v/v) sodium hypochlorite containing the wetting agent 20 for 15 min. After being rinsed 4 times with sterile distilled water, seeds were cultured on modified VW medium (VW medium supplemented with 100 mg L^{-1} myo-inositol, 1 mg L^{-1} thiamine, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine and 4 mg L^{-1} glycine, 15% coconut water and 0.8% agar at pH 5.4). The explants were cultured under 16 h photoperiod (light intensity 40 μ mole m^{-2} sec^{-1}) at 25±2°C for 4 weeks.

Protocorm proliferation: To evaluate the effect of NAA and BA on proliferation of protocorm, four week old protocorms (1 g) were culture on modified VW medium and 0, 0.5 and 1 mg L^{-1} NAA in combination with 0, 1 and 2 mg L^{-1} BA. The cultures were then maintained under 16 h photoperiod (light intensity 40 μmole $m^{-2}$$sec^{-1}$) at 25±2°C for 4 weeks. The growth of protocorms were then recorded.

Effect of antibiotic on plant growth: For determining the effect of antibiotics on the growth of *D. secundum*, hygromycin and cefotaxime were added to the regeneration medium. The concentrations tested were 0, 10, 15, 20, 25 and 30 mg L^{-1} hygromycin and 100, 200, 300, 400 and 500 mg L^{-1} cefotaxime. The antibiotics were added to modified VW medium after autoclaving. Protocorms were cultured at 25°C 16 h photoperiod. After four weeks of culturing, the effectiveness of the antibiotics was evaluated.

Effect of cefotaxime on growth of *A. tumefaciens* LBA 4404 (pCAMBIA 1305.1): *A. tumefaciens* strain LBA 4404 (pCAMBIA 1305.1) was cultured in LB liquid medium supplemented with 100 mg L^{-1} kanamycin and 0, 25, 50, 75, 100, 125, 150 and 175 mg L^{-1} cefotaxime. Bacterial cultures were grown on a reciprocal shaker (150 rpm) at 28°C for 24 h. Bacterial growth was measured at OD_{550}.

Transformation: *A. tumefaciens* strain LBA 4404 (pCAMBIA 1305.1) was used for establishment of the transformation. The plasmid pCAMBIA contained antisense ACC oxidase, β-glucuronidase (GUS) and hygromycin resistance (*hptII*) gene, each of which was expressed under CaMV 35S promoter. Protocorms were used as the explants for transformation.

Effect of co-cultivation condition on transformation efficiency: *A. tumefaciens* strain LBA 4404 (pCAMBIA 1305.1) was cultured in LB liquid medium supplemented with 50 mg L^{-1} kanamycin. Bacterial cultures were grown overnight on a reciprocal shaker at 28°C until the OD_{550} = 1.0. The following day, Agrobacterium suspension supplemented with 200 μM acetosyringone and the explants were soaked in Agrobacterium suspension for 10, 20, 30, 40, 50 and 60 min. The protocorms were then collected and transferred to sterile filter paper for absorbtion of excess Agrobacterium and transferred to solidified VW_2 medium. After 3 days of co-cultivation, the protocorms were washed with sterile distilled water containing cefotaxime. Explants co-cultivated with *A. tumefaciens* were transferred to VW_2 medium and supplemented with cefotaxime and hygromycin after autoclaving. The optimal co-cultivation time for transformation efficiency was determined by recording numbers of blue spots on explants.

Agrobacterium-mediated transformation: Protocorms were immersed in the Agrobacterium suspension supplemented with 200 μM acetosyringone for appropriate inoculation time, then transferred to sterile filter paper for absorption of excess Agrobacterium. They

were then co-cultivated on modified VW medium for three days. After co-cultivation, the protocorms were washed thoroughly in sterile distilled water containing cefotaxime. Explants co-cultivated with *A. tumefaciens* were transferred for selection procedure to the modified VW medium supplemented with cefotaxime and hygromycin after autoclaving. The surviving protocorms were then transferred to the regeneration medium supplemented with the same selective agents.

Assay for β-glucuronidase (GUS) activity: The histochemical assay for GUS gene expression was performed according to the method of Jefferson (1987) Plant tissue were immerse in X-gluc (5- bromo-4- choro-3-indoyl glucoronide) solution and incubated overnight at 37°C. After staining, the materials were treated with 70% ethanol to remove chlorophyll before observation. Transient GUS expression efficiency of protocorms was examined.

PCR analysis: Total genomic DNA was extracted from both the transformed protocorms and non transformed (control) protocorms by the DNeasy® Plant Mini kit (Qiagen). The primer sequences for PCR were as follows: 35 S forward sequence (F)5'GCTCCTACAAA TGCCATCA-3', reverse sequence (R) 5'GATAGTGG GATTGTGCGTA-3' to yield a 195 bp fragment; NOS (F) 5'GAATCCTGTTGCCGGTCTTG-3' (R) 5' TTATCCTA GTTTGCGC GCTA-3' to yield a 180 bp and ACC (F) 5' CGCGGATCCGCNTGYSARAANTGGGGNTT 3' (R) 5' CGCGGATCCGCNTGYSARAANTGGGGNTT 3' to yield a 843 bp fragment. For 35S, the DNA was predenatured at 94°C for 5 min followed by 25 cycles of amplification (94°C 30 sec^{-1}, 55°C/0 sec^{-1}, 72°C 30 sec^{-1}). The final extension step at 72°C for 7 min. For NOS, the DNA was predenatured at 94°C for 5 min followed by 40 cycles of amplification (94°C 20 $sec,^{-1}$ 60°C 40 sec^{-1}, 72°C/1 min). The final extension step at 72°C for 10 min. For ACC, the DNA was predenatured at 94°C for 5 min followed by 35 cycles of amplification (94°C 20 sec^{-1}, 55°C 30 sec^{-1}, 72°C 30 sec^{-1}). The final extension step at 72°C for 5 min. The reaction material was cooled and kept at 4°C. The PCR products were visualized by running the completed reaction on 1.5% agarose gel containing ethidium bromide.

RESULTS

Protocorm induction and protocorm proliferation: Seeds were germinated at 30 days after the initiation of culturing and developed to protocorms at 45 days after germination on modified VW medium. High proliferation capacity of

Table 1: Effect of different combination of NAA and BA on protocorm proliferation of *D. secundum* on VW medium containing 100 mg L^{-1} myo-inositol, 1 mg L^{-1} hiamine, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine, 4 mg L^{-1} glycine

Growth regulator (mg L^{-1})		
NAA	BA	Protocorm weight (g) mean±SE
0.0	0.0	7.74±0.275^{ab}
0.0	0.5	5.68±0.883^{ab}
0.0	1.0	3.33±0.474^{a}
0.5	0.0	12.53±2.537^{c}
0.5	0.5	7.95±2.223^{ab}
0.5	1.0	5.40±1.289^{ab}
1.0	0.0	7.30±2.072^{ab}
1.0	0.5	8.34±1.455^{bc}
1.0	1.0	5.56±1.779^{ab}

Means of 5 replicates with the same letter(s) are not significantly different at $p<0.05$ (DMRT)

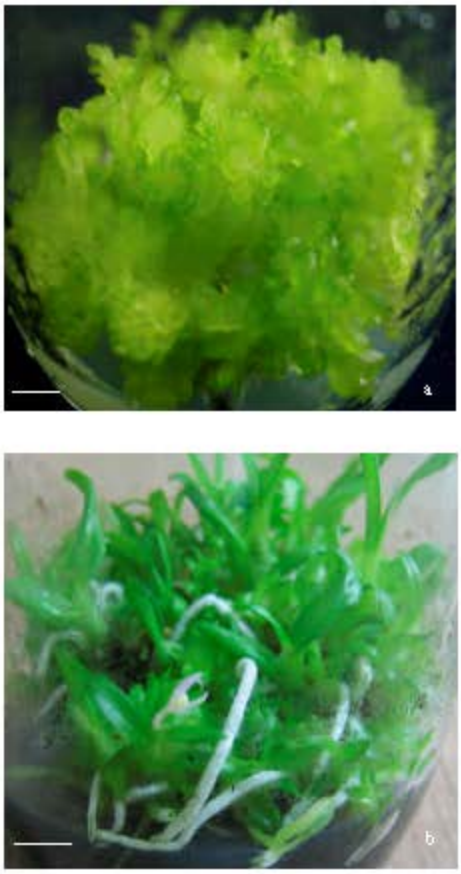

Fig. 1: (a) Protocorms of *D. secundum* cultured on VW medium containing 100 mg L^{-1} myoinositol, 1 mg L^{-1} thiamine, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine, 4 mg L^{-1} glycine and 0.5 mg L^{-1} NAA. (b) Plantlets of *D. secundum* (— = 5 mm)

D. secundum protocorms were observed two months after being cultured on modified VW medium supplemented with 0.5 mg L^{-1} NAA (12.53±2.537 g) (Table 1 and Fig. 1).

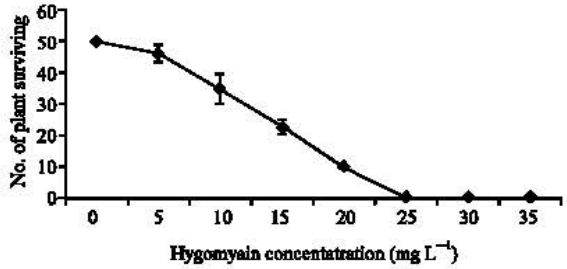

Fig. 2: Effect of hygomycin on growth of *D. secundum*

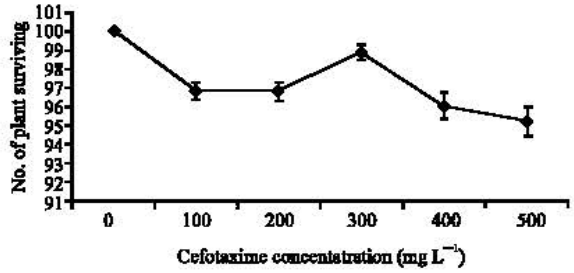

Fig. 3: Effect of cefotaxime on growth of *D. secundum*

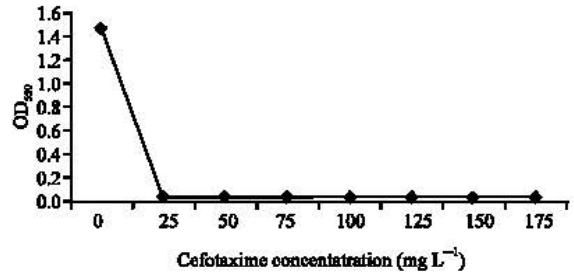

Fig. 4: Effect of cefotaxime on growth of *A. tumefaciens* LBA 4404 (pCAMBIA 1305.1)

Effect of antibiotic on growth of *D. secundum*: In the presence of 10-30 mg L^{-1} hygromycin and 100-500 mg L^{-1} cefotaxime, a slightly inhibition effect was observed. The lowest dose of hygromycin that completely inhibited protocorms growth was 25 mg L^{-1} (Fig. 2). All of the protocorms turned brown and eventually died in 4 weeks after they were transferred to the selective medium. The highest dose of cefotaxime that yielded surviving protocorms was 500 mg L^{-1} (Fig. 3).

Effect of cefotaxime on the growth of *A. tumefaciens* LBA 4404 (pCAMBIA 1305.1): *A. tumefaciens* strain LBA 4404 (pCAMBIA 1305.1) showed good growth in LB medium without cefotaxime (OD_{550} = 1.47). The lowest dose of cefotaxime that inhibited bacterial growth was 50 mg L^{-1} (OD_{550} = 0.028). The optical densities of bacterial culture in LB media that contained 75, 100, 125, 150 and 175 mg L^{-1} cefotaxime were 0.026, 0.023, 0.018, 0.017 and 0.016, respectively (Fig. 4).

Transformation: β-glucuronidase activity was used to monitor transformation efficiency. Blue staining was observed after seven days of co-cultivation. The highest number of blue spots was observed from protocorms co-cultivated in Agrobacterial suspension for 40 min (Table 2 and Fig. 5). DNA integration of the transformed protocorms was investigated using the PCR method. It was found that the sizes of amplified fragments were 195, 180 and 843 bp for the 35S, NOS and antisense ACC oxidase respectively, whereas the non-transformed control orchid did not show any of the expected band sizes (Fig. 6 and 7).

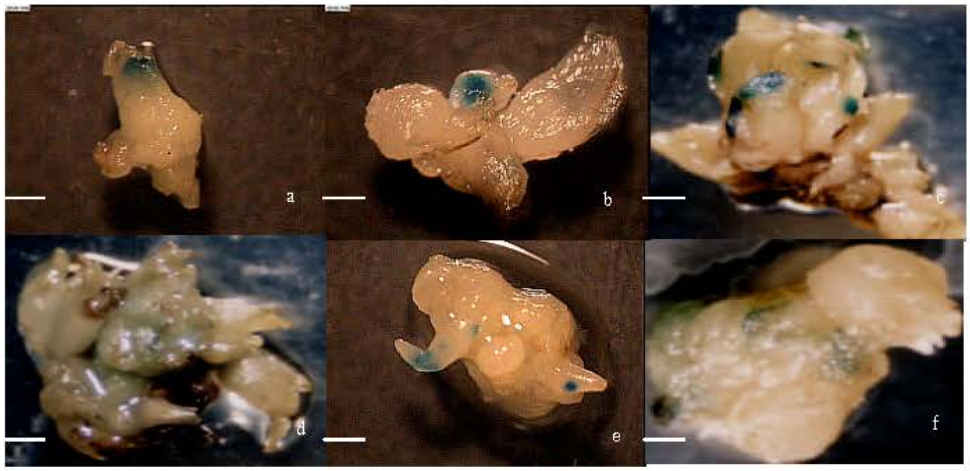

Fig. 5: Visual detection of histochemical staining for GUS activity in the transgenic protocorms and plantlets of *D. secundum* by using Agrobacterium-mediated transformation. (a-f) Incubation in Agrobacterium suspension for 10, 20, 30, 40, 50 and 60 min, respectively (— = 0.2 mm)

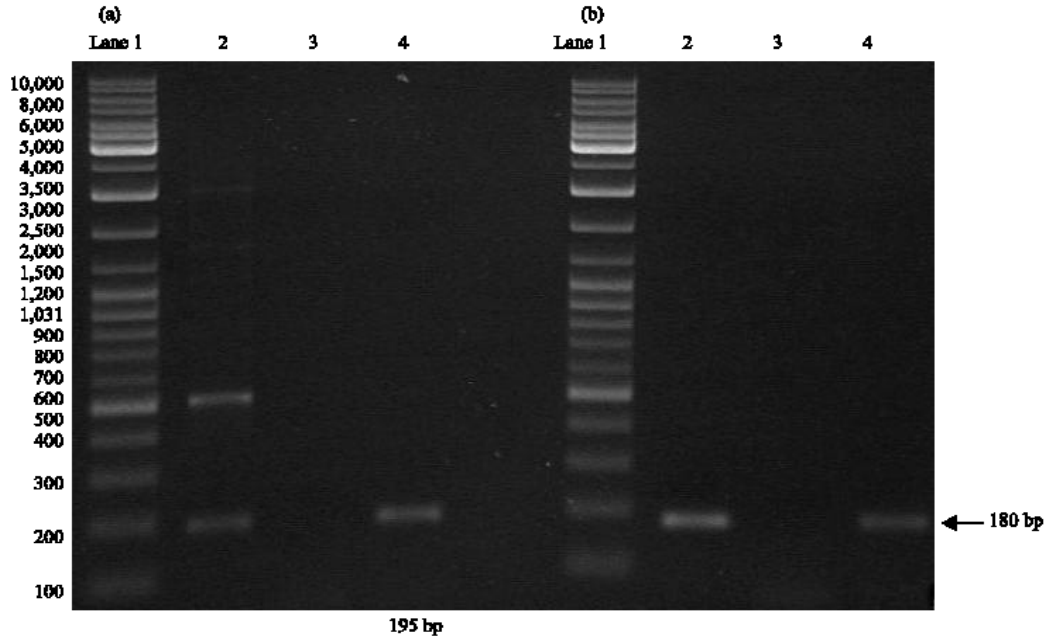

Fig. 6: PCR analysis of transformed protocorms of *D. secundum* using primers to detect the 35S (a) and the NOS (b); Lane 1: DNA ladder mix, Lane 2: pCAMBIA 1305.1 containing antisense ACC oxidase gene, Lane 3: non transformed protocorms Lane 4: hygromycin resistant protocorms

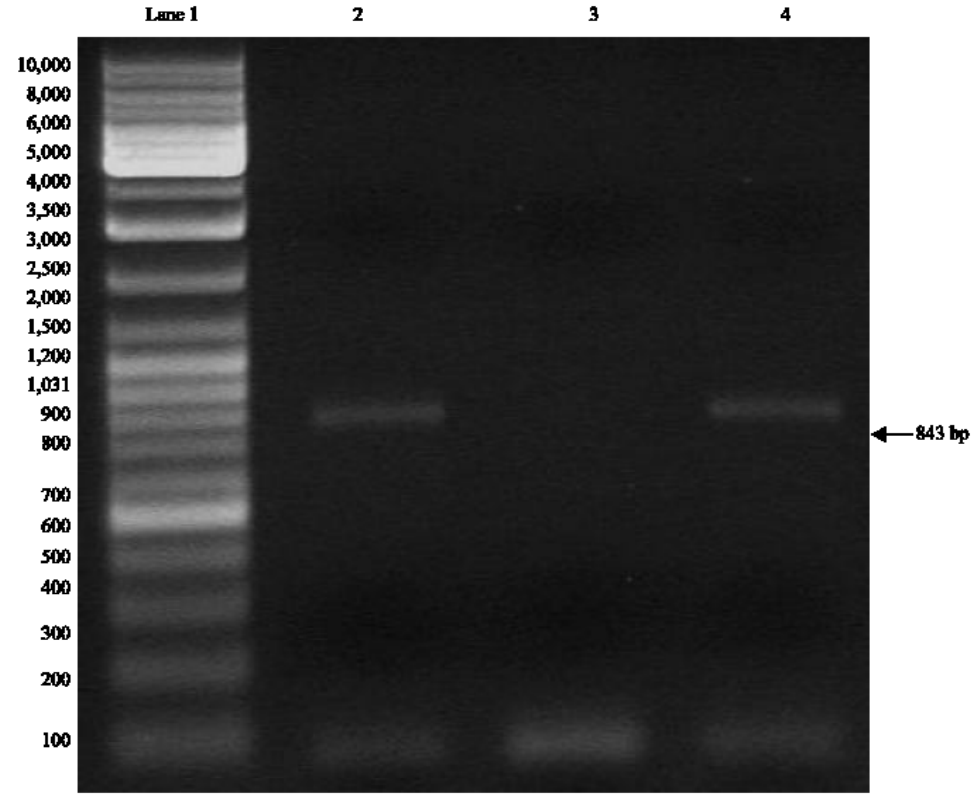

Fig. 7: PCR analysis of transformed protocorms of *D. secundum* using primers to detect the antisense ACC oxidase; Lane 1: DNA ladder mix, Lane 2: pCAMBIA 1305.1 containing antisense ACC oxidase gene, Lane 3: non transformed protocorms and Lane 4: hygromycin resistant protocorms

Table 2: Effect of cocultivation in *A. tumefaciens* LBA4404 (pCAMBIA1305.1) on transient GUS assay of protocorms

Co-cultivation time (min)	No. of protocorms	GUS assay±SE (%)
10	50	22.6±1.45[a]
20	50	27.6±1.2[b]
30	50	33.0±0.577[c]
40	50	60.0±0.577[e]
50	50	38.0±0.577[d]
60	50	29.0±0.667[b]

Means of 3 replicates with the same letter(s) are not significantly different at $p<0.05$ (DMRT)

DISCUSSION

The specific combination of auxin and cytokinin in culture media is one of the most important factors for *in vitro* plant regeneration of orchid. Generally, NAA is the most effective auxin for protocorm proliferation of *D. secundum*. In this study, we found that the most suitable auxin for protocorm proliferation was 0.5 mg L^{-1}. Such results were found that NAA alone could induce PLBs from the culture shoot tips of Doritaenopsis (Tokuhara and Mii, 1993) and addition of BA concentration at a low concentration (0.4 mM) completely inhibited PLBs formation of Phalaenopsis orchid (Tokuhara and Mii, 2003).

A selective agent is crucial for selection of the transformants in distinguishing the appropriate medium. Hygromycin is a widely used as a selective reagent in monocotyledonous transformation. In orchids, hygromycin also successfully discriminates between transformed and nontransformed cells (Belarmino and Mii, 2000). In this study, we found that hygromycin at 25 mg L^{-1} worked well for the selection of the transformants. This antibiotic concentration is even higher than that used in Pipatpanukul *et al*. (2004) and Mishiba *et al*. (2005) who use hygromycin at 20 mg L^{-1} as a selective agent of rice cv. RD6 and Phalaenopsis orchid, respectively. And this is even lower than that used in Men *et al*. (2003) who use hygromycin at 30 mg L^{-1} as a selective agent of *Dendrobium phalaenopsis* and *D. nobile*. Yu *et al*. (1999), Jeknic *et al*. (1999) and Belarmino and Mii (2000) who reported that hygromycin at 50 mg L^{-1} completely prevented the growth of non-transformed plants of *Dendrobium hybrid* MiHua, *Iris germanica* and Phalaenopsis orchid, respectively.

In the transformation of plants, co-cultivation period of explants with an Agrobacterium culture, elimination of Agrobacterium with antibiotics is required. Several samples of the uses of antibiotics such as carbenicillin at 50 mg L^{-1} (Yu *et al*., 2001), timentin at 200 mg L^{-1} (Liau *et al*., 2003) meropenem at 5 mg L^{-1} (Mishiba *et al*., 2005) and cefotaxime at 200-500 mg L^{-1} (Jeknic *et al*., 1999; Belarmino and Mii, 2000; Men *et al*., 2003; Pipatpanakul *et al*., 2004) have been studied. Cefotaxime has been used regularly in Agrobacterium-mediated transformation of crops in order to suppress or eliminate Agrobacterium. It kill bacteria by interfering with the ability of bacteria to form cell walls. When this happens, the bacteria were degraded and died. Cefotaxime worked well in Agrobacterium-mediated transformation and is commonly used for excluding Agrobacterium tumefaciens during plant transformation (Yu *et al*., 2001). Men *et al*. (2003) reported that co-cultured Dendrobium protocorms with Agrobacterium for 2-3 days and transferred to selective medium containing 300 mg L^{-1} cefotaxime (in order to inhibit the growth of A. tumefaciens) proved to be simple and efficient.

Co-cultivation period is a factor which affects efficiency of Agrobacterium mediated transformation. The co-cultivation time with Agrobacterium ranges from 1-2 min to 3 days depending on plant species (Mendel and Hansch, 1995). Men *et al*. (2003) transformed *Dendrobium nobile* by immersed in the *A. tumefaciens* suspension for 30 min followed by co-cultivation on 1/2 MS medium with 1 mg L^{-1} BA and 100 μM acetosyringone (or without acetosyringone) for 2-3 days gave the highest transformation efficiency. This is not the similar to present result. The optimal co-cultivation time for *D. secundum* was 40 min and then incubation them on the modified VW medium for 3 days. These transformed protocorms gave high intensity of blue stain that indicated high GUS expression. Furthermore, An external supply of 200 μM acetosyringone during co-cultivation is essential for pre-activated *A. tumefaciens*. This phenolic compound is sufficient for activation of vir genes to initiate T-DNA transfer (Stachel *et al*., 1985). Transformation in the orchid Phalaenopsis, extending the co-cultivation period up to 5-7 days did not increase GUS transient expression activity, but caused necrosis and death of cells (Belarmino and Mii, 2000). It is indicated that a long co-cultivation period can significantly reduce transformation efficiency.

The presence of the transient gene in putatively transformed plants was confirmed by PCR amplification of a 195 and 180 bp fragment of the CaMV 35S promoter and the NOS terminator. Present research has shown the same result as those seen in *D. draconis* and *Dendrobium miss* Singapore (Homkrajhae, 2005) and as well as in rice (Maneewan *et al*., 2005). Furthermore we were confirmed by PCR amplification of 843 bp of the antisense ACC oxidase gene.

In summary, this report described the use of *A. tumefaciens* strain LBA 4404 (pCAMBIA 1305.1) to transfer antisense ACC oxidase, screenable and selectable marker genes into *D. secundum* and showed molecular

evidence of transgenic plants. We confirmed that protocorms and plantlets of *D. secundum* are suitable target tissues for Agrobacterium-mediated transformation. We also propose that this method can be used to transform novel genes into other orchid species.

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