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Full Length Research Paper

Agrobacterium-mediated transformation of *Dendrobium chrysotoxum* Lindl.

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The aim of this study was to improve the efficiency of plantlet regeneration and to establish an effective protocol for genetic transformation of *Dendrobium chrysotoxum*. Seeds successfully germinated on the ND medium amended with 1% potato extract, and further developed into protocorms with high proliferation when transferred to the ND medium containing 1% sucrose. Protocorms produced the maximum number of seven shoots per protocorm in the presence of 1 mg/l BA. For successful genetic transformation of *D. chrysotoxum* mediated by *Agrobacterium tumefaciens* strain LBA4404 (pCAMBIA 1305.1), carrying *antisense ACC oxidase*, *gus* and *hpt II* genes, the explants were inoculated for 45 min in liquid medium of earlier mentioned bacterial strain followed by co-cultivation and selection on 25 mg/l hygromycin. The results show maximum of 23% transformation. Transformation was confirmed by GUS assay and PCR, indicating successful integration of gene.

Key words: *Dendrobium chrysotoxum*, antisense ACC oxidase, cefotaxime, hygromycin, benzyleaminopurine.

INTRODUCTION

The orchid genus *Dendrobium*, one of the most important commercial plants used as cut flowers and potted plants, belongs to the family Orchidaceae, which is one of the largest plant families with approximately 20,000 species (Dressler, 1993). In Thailand, approximately 177 genera and 1,135 species are found in a variety of habitats (Atichart et al., 2007).

Longevity of vase life is an important factor in consumer preference of cut flowers. Orchid flowers are extremely sensitive to ethylene, the gaseous plant hormone, even at low level. It induces rapid wilting and senescence of flowers and petals, abscission of plant parts including flower structures, and discoloration of flowers (Woodson, 1994). In plant tissues, ethylene is biosynthesized by the conversion of S-adenosyl methionine to 1-aminocyclopropane-1-carboxylic acid (ACC). This reaction is catalyzed by ACC synthase (Kende, 1993). ACC is then converted to ethylene by ACC oxidase. Therefore, senescence is ably prevented by inhibiting the activity of either ACC synthase or ACC oxidase.

To date, plant genetic engineering, providing an opportunity to genetically modify a wide range of organisms, has been introduced to orchid industry in order to produce desirable orchid traits, with considerable protocols developed for a variety of orchid species. For instance, a transgenic *Phalaenopsis* orchid has been successfully produced by means of *Agrobacterium*-mediated transformation (Belarmino and Mill, 2000). This study was therefore carried out in order to find the suitable protocol for transferring an *antisense ACC oxidase* gene into *Dendrobium chrysotoxum* protocorms via *Agrobacterium*-mediated transformation so as to produce the orchid trait ably inactivating the activity of ACC oxidase and enhancing longevity of vase life of orchid flowers.

MATERIALS AND METHODS

Protocorm induction

D. chrysotoxum pods were initially washed with mild detergent and

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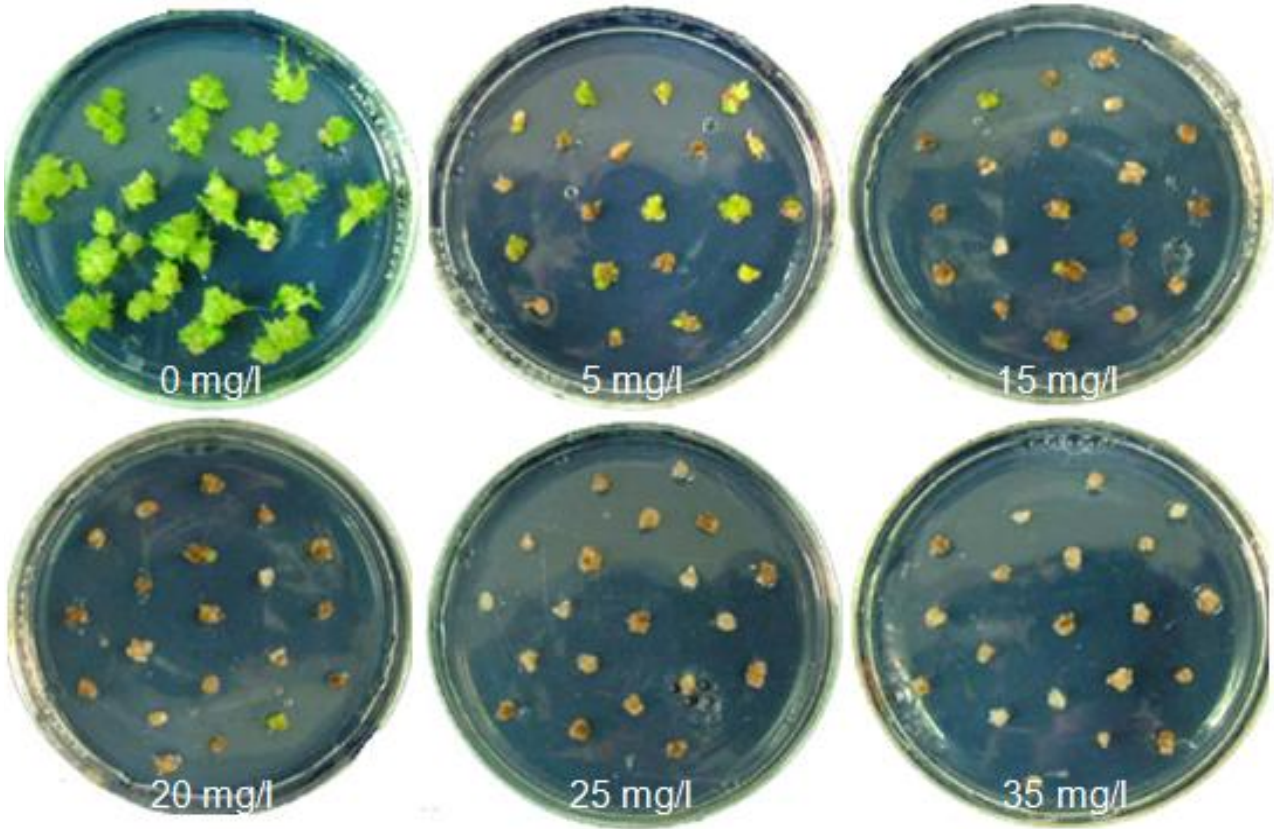

Figure 1. Protocorm development after culture on the ND medium supplemented with varied hygromycin concentrations for four weeks.

then soaked in 15% (v/v) sodium hypochlorite amended with Tween-20 for 15 min. They were subsequently rinsed three times with sterile distilled water. After that, the pods were longitudinally dissected and seeds were picked out. The seeds were sown on the Vacin and Went (VW) medium and the new Dogashima (ND) medium containing 1 or 2% (w/v) sucrose and 1% (w/v) potato extract at pH 5.7. The cultures were maintained under a 16-h photoperiod (a light intensity of 40 μmole $m^{-2}s^{-1}$) at 25 ± 2°C followed by recording of seed germination percentage after four weeks.

Protocorm proliferation

D. chrysotoxum protocorms were cultured on the ND medium containing 0.01 or 1 mg/l 1-naphthaleneacetic acid (NAA) in combination with 0, 0.1, 1 or 5 mg/l BA. The cultures were maintained under a 16-h photoperiod at 25 ± 2°C. After four weeks, average number of shoots per protocorm was determined. The experiment was performed in triplicate.

Selection of putative transformants

Putative transformants were selected by culturing transformed protocorms on a proliferation medium amended with 0, 5, 15, 20, 25 or 35 mg/l hygromycin, under a 16-h light photoperiod using a light intensity of 40 μmole $m^{-2}s^{-1}$ at 25 ± 2°C. After four weeks of culture, percentages of proliferation and number of shoots per protocorm were recorded. The experiment was performed in triplicate.

Transformation of *D.* chrysotoxum

Agrobacterium tumefaciens strain LBA 4404 (pCAMBIA1305.1) containing antisense ACC oxidase, β-glucuronidase (*gus*) and hygromycin-resistant (*hpt II*) genes was used for establishment of a transformation protocol. *A. tumefaciens* was cultured in the YEP liquid medium and grown on a reciprocal shaker at 28°C overnight.

Protocorms were soaked in *Agrobacterium* suspension for 30, 45, 60 or 75 min and then co-cultivated on the ND medium. After three days of co-cultivation, the protocorms were washed thoroughly in sterile distilled water amended with cefotaxime, and subsequently transferred to a proliferation medium supplemented with hygromycin. One month later, transient gene expression was verified by GUS assay by treating protocorms with 5-bromo-4-chloro-3-indolyl glucuronide (x-gluc) at 37°C overnight.

PCR analysis

Total genomic DNA was extracted from transformed- and nontransformed plants by the *cetyltrimethylammonium* bromide (CTAB) method (Doyle and Doyle, 1987). The primer sequences: 35S forward sequence (F) 5'-GCTCCTACAAATGCCATCA-3' and 35S reverse sequence (R) 5' GATAGTGGGATTGTGCGTCA-3'

Table 1. Percentages of seed germination and protocorm induction after culture on the VW and ND media.

Seed germination (%)		Protocorm induction (%)	
VW	ND	VW	ND
100	100	100	100

were employed to yield the 195 bp fragments, while the primer sequences: NOS (F) 5'-GAATCCTGTTGCCGGTCTTG-3' and NOS (R) 5'-TTATCCTAGTTTGCGCGCTA-5' were used to yield the 180 bp fragments. DNA was denatured at 94°C for 4 min, followed by 45 cycles of amplification (1 min at 94°C; 1 min at 45°C, 1 min at 72°C). The reaction material was cooled and kept at 4°C and PCR products were visualized by running the completed reaction on a 1.5% agarose gel containing 0.5 mg/l ethidium bromide.

Statistical analysis

Data were statistically analyzed using one way analysis of variance (one way-ANOVA) and least significant difference (LSD). Values were calculated at p = 0.01 for comparing means of the treatments.

RESULTS

Protocorm induction

Protocorm development was observed after four weeks of seed sowing on the Vacin and Went (VW) and ND media containing 1 or 2% sucrose.

The effects of the VW and ND media on protocorm induction are shown in Table 1. The full strength of both media was effective for protocorm induction. The results were similar to those reported by Tokuhara and Mii (1993) stating that the ND medium performed the best for seed germination and protocorm induction of most orchid species.

Protocorm proliferation

Since protocorm induction was achieved at the initiation of cultures, further investigation was aimed at optimizing plantlet production. Five treatments with varied concentrations of NAA and BA were tested. Two-month protocorms were cultured on the ND medium containing NAA and BA. The highest shoot number per protocorm was seven when cultured on the ND medium enriched with 1 mg/l BA (data not shown).

Selection of putative transformants

Hygromycin strongly reduced protocorm proliferation. Protocorm development was completely inhibited in the presence of 25 mg/l hygromycin (Figure 1). Treated protocorms turned brown in color and eventually died within three weeks.

Transformation of *D. chrysotoxum*

β-Glucuronidase enzyme activity was used to examine transformation efficiency. Blue coloration was observed after five days of inoculation. Viable protocorms, aged 1 month, showed stable expression of *gus* activities as shown in Figure 2. The optimal inoculation period was 45 min (Figure 3).

PCR analysis

DNA integration of transformed plants was investigated using PCR method. We found that the sizes of amplified fragments were 195 and 180 bp for the 35S and NOS, respectively, whereas untransformed control orchids did not show any band size (Figure 4).

DISCUSSION

Plant growth regulators are considered important for growth promotion of many plant species. They are required either singly or combined. According to this study, BA concentration of 1 mg/l was considered as the most suitable dose for the transformation of *D. chrysotoxum*. These results are in agreement with Arditti and Ernst (1993), stating that amendment of a suitable amount of plant growth regulators in the culture medium enhanced the efficiency of protocorm proliferation. A variety of antibiotics are used in many transformation systems as a selective agent. In monocot transformation systems, hygromycin is widely employed. A selective agent is very crucial for transformant selection due to its property to prevent development of undesirable escape plants (Datta et al., 1999). In our experiments, an *hpt II* gene was employed as a selectable marker. Hygromycin is an aminoglycoside antibiotic which causes death to plant cells by inhibiting the progress of transcription and translation. Moreover, hygromycin is suggested as an excellent selectable agent, and its optimal dose varies among plant species (Cheng et al., 1998; Datta et al., 1999). Our findings showed that 25 mg/l hygromycin was

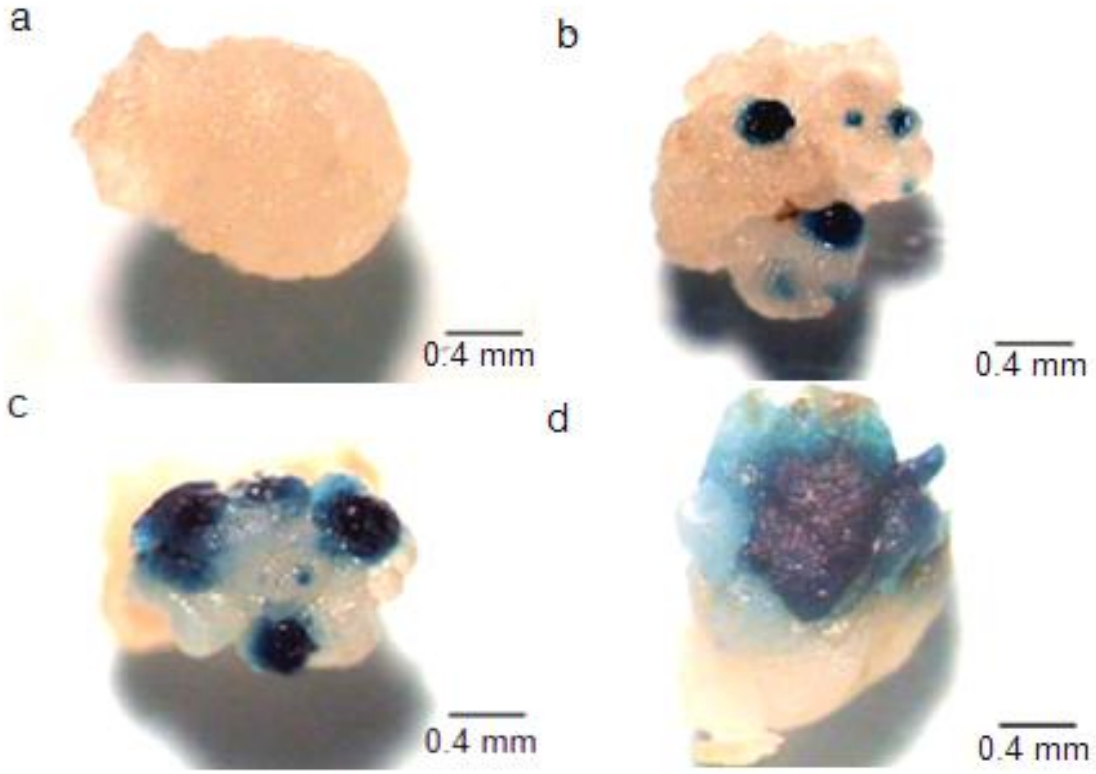

Figure 2. Levels of transient gene expression assessed by histochemical GUS assay. a) Control; b) low level; c) moderate level; d) high level.

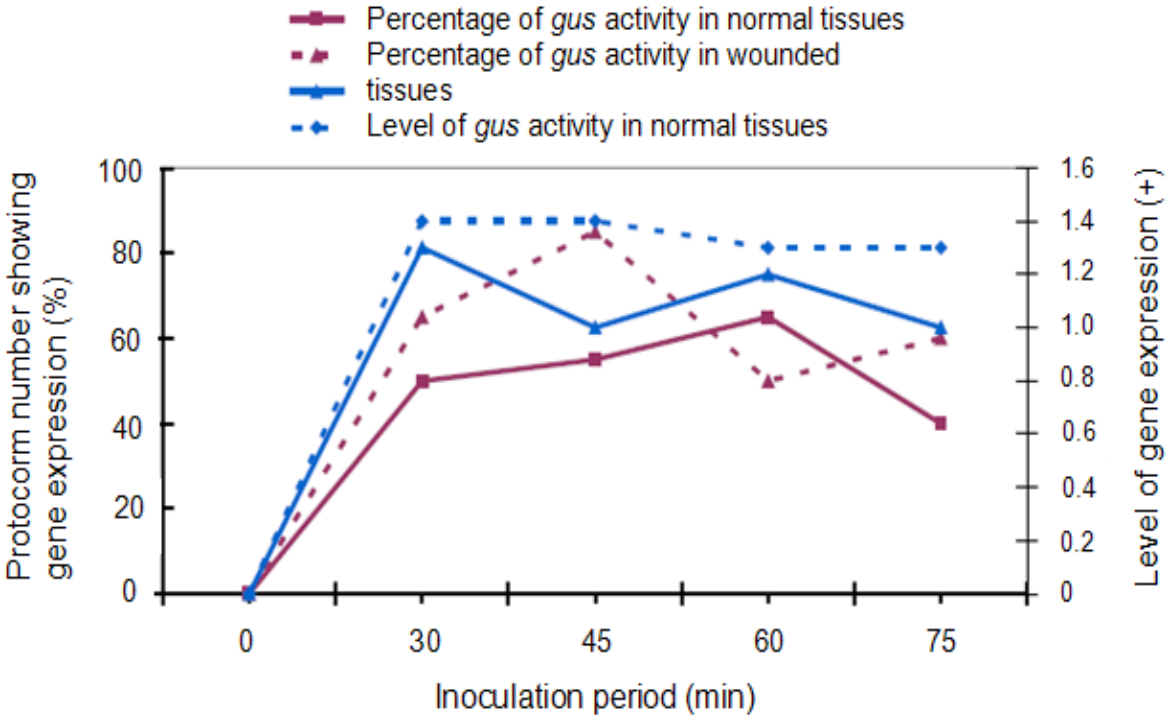

Figure 3. Percentage and levels of *gus* activity.

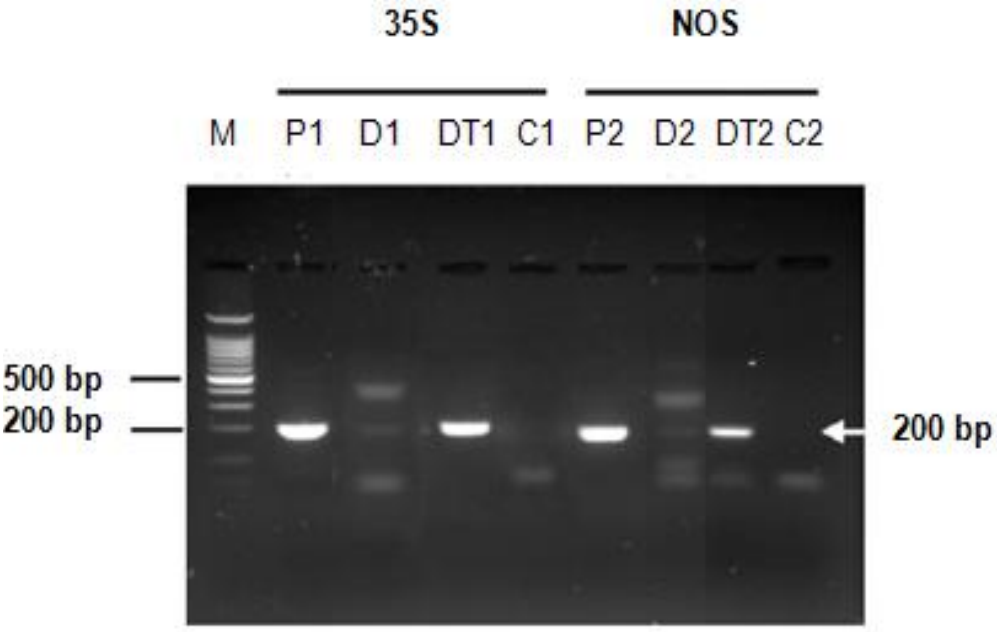

Figure 4. Evaluation of gene integration by PCR technique using the primer 35S in association with anti-35S (lanes, P1 to C1), and the primer NOS in association with anti-NOS (lanes, P2 to C2). Lane M = 100 bp DNA ladder; P = the plasmid pCAMBIA1305.1; D = nontransformed protocorms; DT = transformed protocorms; C = control.

effective for transgenic selection.

Inoculation of explants with *A. tumefaciens* is a key factor that determines the success in genetic transformation. The inoculation period influences the efficiency of the transformation system mediated by *Agrobacterium* (Men et al., 2003), and it varies among plant species (Mendel and Mansch, 1995). In our study, the optimal inoculation period was 45 min, giving rise to the highest transient gene expression. In general, PCR was used to confirm the integration of genes of interest into plant genome besides prescreening by transient gene expression. Based on these findings, the PCR analysis showed detection of *hpt* and *gus* gene fragments. The sizes of amplified fragments were 195 and 180 bp for the 35S and NOS, respectively.

In summary, an efficient transformation protocol for the species *D. chrysotoxum* was successfully established and was guaranteed by the molecular evidence of transgene integration into the orchid genome. Therefore, this protocol might be used to develop transgenic orchids which enhance longevity of their flowers.

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