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Agrobacterium-mediated transformation of *Dendrobium* orchid with the flavanone 3-hydroxylase gene

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Abstract: The *Ascocenda* flavanone 3-hydroxylase (*AcF3H*) gene was successfully transformed into *Dendrobium* 5N white orchid plants using *Agrobacterium*-mediated gene transformation. In this study, for the first time, we report the construction of a plant expression vector harboring the *AcF3H* gene using the Gateway cloning system. Protocorm-like bodies (PLBs) were cocultivated with the *Agrobacterium tumefaciens* strain AGL1 harboring the plant expression vector pGWB5-*AcF3H*, which contained the hygromycin phosphotransferase (*hpt*) gene as a selectable marker. The highest transformation efficiency (10.13%) was achieved when PLBs were cocultivated with *Agrobacterium* cells for 15 min. Three months after the transformation, the plantlets were regenerated, and the transgenic plants were confirmed by polymerase chain reaction (PCR) analysis using specific primers for the *hpt* gene and 35S promoter region. PCR products of approximately 400 and 500 bp, corresponding to the *hpt* gene and the *35S* promoter, respectively, were detected in the transgenic plants, while no such product was observed in nontransgenic plants, indicating that the *AcF3H* gene was integrated into the genome of *Dendrobium* 5N white orchid plants. The transient expression of the *AcF3H* gene in *Dendrobium* 5N white and *Dendrobium* Anna petals was performed using the agroinfiltration technique, and the results demonstrated that no cyanidin content was detected in the *Dendrobium* 5N white petals after *AcF3H* infiltration. In contrast, the cyanidin content was increased by approximately 6% in the *Dendrobium* Anna petals, suggesting that the *AcF3H* gene was transiently expressed in this orchid.

Key words: Flavanone 3-hydroxylase, *Dendrobium* orchid, *Agrobacterium tumefaciens*, protocorm-like bodies

1. Introduction

Dendrobium orchids are economically important ornamental plants in the cut flower industry of Thailand. These orchids exhibit the desirable characteristics of perfectly shaped flowers and have a long vase life. Among the different *Dendrobium* hybrid varieties, *D. phalaenopsis* has become the signature Thai *Dendrobium*, with round, full, and flat flower faces 8 cm wide or larger (Kuehnle, 1997). Due to the narrow range of flower color, the modification of new orchid varieties with a wide range of flower color has been an area of recent focus. During the past decade the development of new orchid varieties has been limited to only the conventional breeding method, which is time- and labor-consuming. Currently, biotechnological approaches, particularly genetic engineering techniques, are being extensively studied for new plant development, and one of the key benefits of using these techniques is the molecular information gained, especially on genes involved in the anthocyanin biosynthetic pathway.

Anthocyanins, the main source of flower color, are derived from anthocyanidins by the addition of sugar molecules. Three major well-known anthocyanidins are cyanidin (red color), pelargonidin (orange color), and delphinidin (blue color). The generalized anthocyanin biosynthesis pathway is well established, and there are many genes involved in this pathway (Holton and Cornish, 1995; Tanaka et al., 2008, 2009).

In higher plants, flavanone 3-hydroxylase (F3H) is a crucial enzyme involved in the anthocyanin biosynthesis pathway. It catalyzes the conversion of naringenin to dihydrokaempferol, which is the central intermediate in the anthocyanidin biosynthesis pathway (Prescott and John, 1996). Genes encoding the F3H proteins (*F3H* genes) have been cloned and characterized from many plant species, such as *Phyllanthus emblica* (Kumar et al., 2015), *Artemisia*

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annua (Xiong et al., 2016), *Lycium chinense* (Song et al., 2016), *Carthamus tinctorius* (Tu et al., 2016), and *Ascocenda* orchid (Khumkarjorn et al., 2016). However, less is known about the molecular cloning and transferring of the *F3H* gene into orchids. In previous studies, the *L. chinense F3H*, *Camellia sinensis F3H*, and tomato *F3H*-like protein genes have been cloned and overexpressed in tobacco (Mahajan and Yadav, 2014; Meng et al., 2015; Song et al., 2016). The foreign gentian *F3H* gene has also been cloned, characterized, and transformed into the white-flowered *Torenia* (Nishihara et al., 2014). Furthermore, suppression of the *F3H* gene in carnation and strawberry has also been carried out (Zuker et al., 2002; Jiang et al., 2013).

Transformation of a foreign gene into orchids to develop new desirable traits is infrequent. This is probably due to the difficulty in constructing the plant expression vector. Since most plant expression vectors are large and have many restriction enzyme recognition sites, the construction of expression vectors is very complicated, time-consuming, and laborious. In recent years, a new series of Gateway binary vectors (pGWBs) for plant transformation has been developed and provides a fast and reliable alternative approach (Nakagawa et al., 2007). Thus, the Gateway cloning system may be an alternative for transformation of the orchid plant with foreign genes.

In this study we report for the first time the construction of a plant expression vector harboring the *Ascocenda F3H* (*AcF3H*) gene using the Gateway cloning system. Transformation of the resulting plant expression vector into the economically important cut flower *Dendrobium* 5N white orchid by *Agrobacterium*-mediated gene transformation was performed and described in this study. The effects of some factors on the transformation efficiency such as the multiplication medium for the protocorm-like bodies (PLBs) and the inoculation time were evaluated. Furthermore, transient expression analysis of the *AcF3H* gene in *Dendrobium* orchids using the agroinfiltration technique was also described.

2. Materials and methods

2.1. Plant materials

PLBs of the *Dendrobium* 5N white orchid plants obtained from Bangkok Green Co., Ltd. were used for genetic transformation. These PLBs were induced from shoot-tip explants of *Dendrobium* 5N white orchid plants using the method described by Saiprasad et al. (2004). Propagation of PLBs was performed by growing the PLBs in 50 mL of liquid modified VW medium (pH 5.2) (Vacin and Went, 1949) supplemented with 15% (v/v) coconut water and 10% (v/v) potato extract. PLBs were cultured in an incubator shaker at 100 rpm and 25 ± 2 °C with an 8/16-h (light/dark) photoperiod. The PLBs were subcultured onto fresh medium every month.

2.2. Optimization of the solid multiplication medium for PLB proliferation

PLBs were grown on solid modified VW medium supplemented with 15% (v/v) coconut water and 6 g/L agar. The effect of sucrose and potato homogenate (PH) on PLB proliferation was studied using a 3^2 factorial in a completely randomized design with three different sucrose concentrations (0, 20, and 40 g/L) and three levels of PH (0, 50, and 100 g/L). One gram of PLBs was cultured in 30 mL of medium in glass bottle of 237 mL at 25 °C with an 8/16-h (light/dark) photoperiod. Each treatment was performed in triplicate and the fresh weight of PLBs was measured at certain time intervals. The data were collected and analyzed using one-way ANOVA and the mean difference from each treatment was analyzed using Duncan's multiple range test (IBM SPSS Statistical Software, version 19).

2.3. Optimum concentration of hygromycin for screening the transgenic plants

The optimum concentration of hygromycin for screening the transgenic *Dendrobium* 5N white plants was evaluated. PLBs approximately 0.4 to 0.6 cm in size were grown on solid modified VW medium containing 0, 10, 20, 30, 40, or 50 mg/L hygromycin. Each treatment was performed in triplicate, and the percentage of PLB survival was calculated after 1 month of cultivation.

2.4. Construction of plant expression vector pGWB5-*AcF3H*

The full-length cDNA of the *Ascocenda F3H* gene (GenBank Accession No. JX392322) was used to construct the plant expression vector pGWB5-*AcF3H*. The recombination sites (*att*) *att*B1 and *att*B2 were added to the *AcF3H* gene by PCR using PrimeSTAR®HS DNA polymerase (Takara, Japan). The primers used were ATT-F3H-F: 5'-GGGG*ACAAGTTTGTACAAAAAA GCA GGCT*CGATGGCGCCTGTTCCGTTCCT-3'(italic letters represent the *att*B1 site)and ATT-F3H-R:5'-GGGG*ACCACTTTGTACAAGAAAGCTGGGT*CT TAAG CTAAAATCTCATTTA-3'(italic letters represent the *att*B2 site). The PCR mixture contained 0.4 µM of each primer, 1X PS buffer, 0.2 mM of each dNTP, 1.25 U of PrimeSTAR®HS DNA polymerase, and 50 ng of template DNA (the pGEM T-easy vector harboring the *AcF3H* gene). PCR amplification was performed at 98 °C for 1 min followed by 30 cycles of 98 °C for 10 sec, 50 °C for 10 s, and 72 °C for 1.5 min.

The entry and plant expression clones were constructed using Gateway technology (Invitrogen, USA). The outline of the construction of the expression vector harboring *AcF3H* is shown in Figure 1. The *att*B-flanked *F3H* gene was introduced into the *att*P-containing donor vector, pDNOR201, by a BP reaction to generate an *att*L-containing entry clone, pDONR201-*AcF3H*. The BP reaction mixture contained 1 µL of *att*B-PCR product (~15–150 ng), 0.5 µL

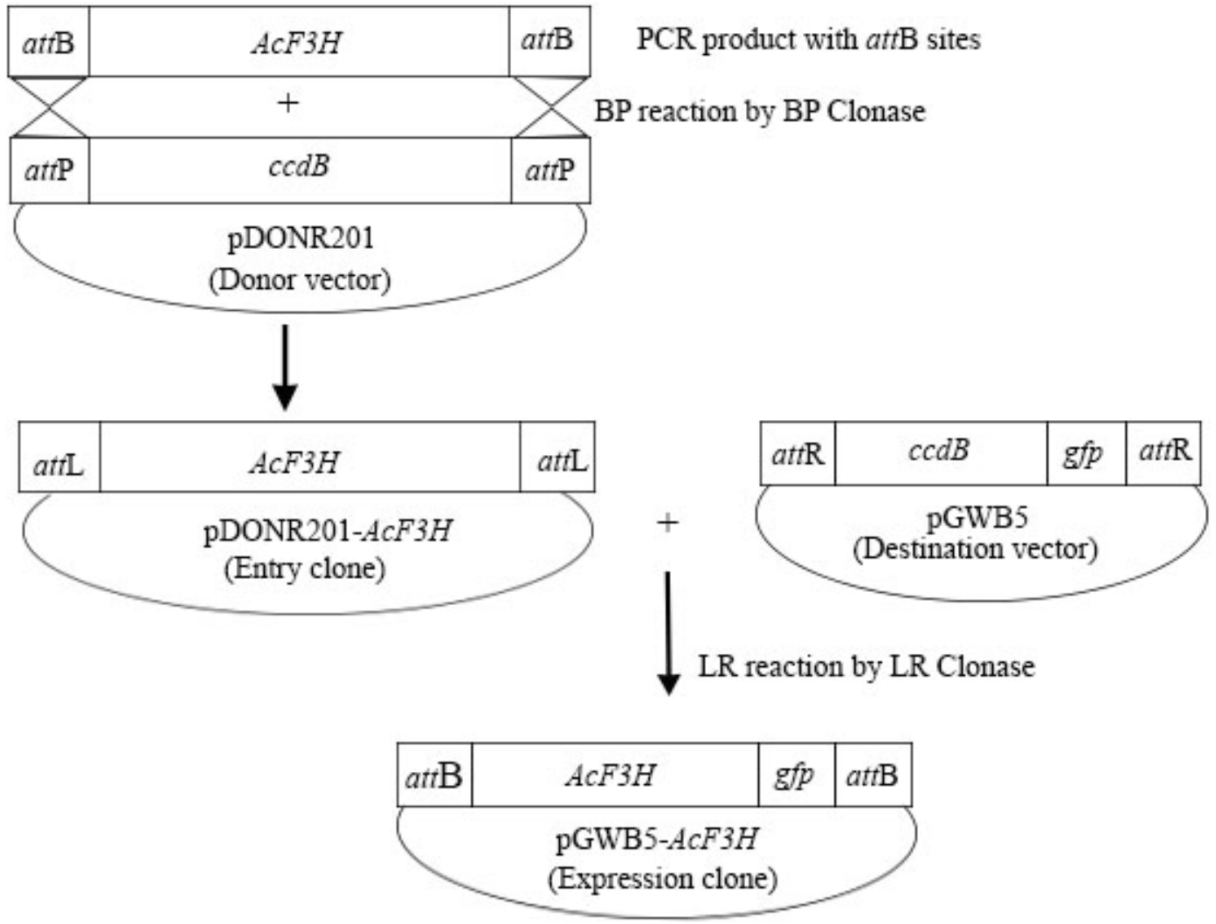

Figure 1. Construction of plant expression plasmid pGWB5-*AcF3H* using the Gateway cloning system (Invitrogen, USA). *AcF3H*, *Ascocenda* flavanone 3-hydroxylase; *att*, recombination site; *gfp*, green fluorescent protein; *ccdB*, control of cell death B gene; PCR, polymerase chain reaction.

of pDONR201 vector (150 ng/µL of supercoiled DNA), 4 µL of TE buffer (pH 8.0), and 1 µL of BP Clonase II enzyme mix. The resulting reaction solution was incubated at 25 °C overnight and then stopped by adding 0.5 µL of 2 µg/µL Proteinase K solution and incubating the mixture at 37 °C for 10 min. Thereafter, 2 µL of the recombination mixture was transformed into *E. coli* DH5α competent cells (50 µL) (Toyobo, Japan) by heat shock at 42 °C for 30 s. The cells were then incubated on ice for 2 min. After adding 450 µL of SOC medium, the culture was incubated at 37 °C for 2 h at 100 rpm. Positive clones were screened on LB medium containing 25 µg/mL of kanamycin. The recombinant plasmid in the selected positive clones was isolated and the presence of the *AcF3H* gene in the recombinant plasmid was confirmed by PCR and DNA sequencing.

The *att*L-containing *AcF3H* gene from the entry clone was transferred to the *att*R-containing destination vector pGWB5 (Nakagawa et al., 2007) by a LR reaction to generate the expression vector pGWB5-*AcF3H*. This vector contained the *hpt* gene as a selectable marker and green florescent protein (*gfp*) as a reporter gene. The expression of the *AcF3H* gene was under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The T-DNA region of the vector is illustrated in Figure 2. The LR reaction consisted of 1 µL of entry clone (50–150 ng/µL of supercoiled DNA), 0.5 µL of destination vector (150 ng/µL of supercoiled DNA), 1 µL of LR Clonase II enzyme mix, and 2.5 µL of TE buffer (pH 8.0). The resulting reaction solution was incubated at 25 °C overnight and then stopped by adding 0.5 µL of 2 µg/µL Proteinase K solution and incubating the mixture at 37 °C for 10 min. The expression vector was transformed into *E. coli* DH5α cells, and the transformants were screened on LB medium supplemented with 25 µg/mL of hygromycin and 25 µg/mL of kanamycin. The expression vector was extracted from the selected transformants and the presence of the *AcF3H* gene in the expression vector was analyzed by restriction digestion and confirmed by PCR and DNA

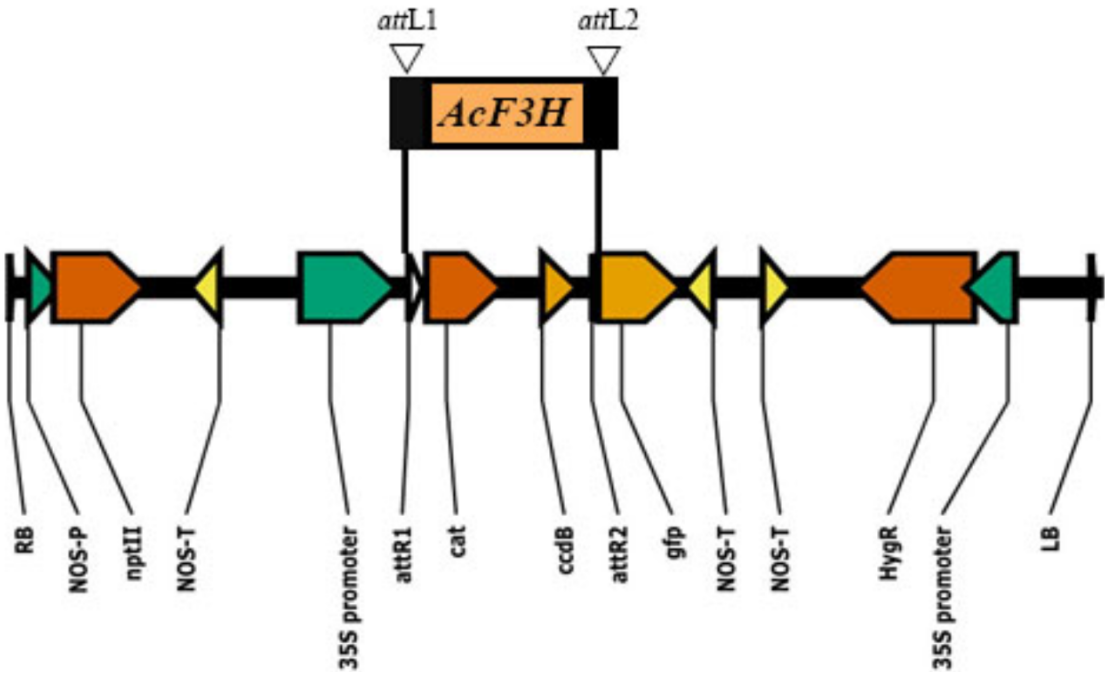

Figure 2. The *AcF3H* gene was transferred into the pGWB5 vector at the *att*R site by an LR reaction. The genes in the T-DNA region of the pGWB5 plasmid are shown above (Nakagawa et al., 2007). RB, right border; NOS-P, nopaline synthase promoter; *npt*II, neomycin phosphotransferase II; 35S promoter, CaMV35S promoter; *att*R1, recombination site R1; *cat*, chloramphenicol acetyltransferase; *ccd*B, control of cell death B gene; *att*R2, recombination site R2; *gfp*, green fluorescent protein; NOS-T, nopaline synthase terminator; *HygR*, hygromycin resistance gene (or *hpt*, hygromycin phosphotransferase gene); LB, left border; *AcF3H*, *Ascocenda* flavanone 3-hydroxylase; *att*L1, recombination site L1; *att*L2, recombination site L2. This map was drawn using the Plasma DNA program (Angers-Loustau et al., 2007).

sequencing. The isolated expression vector harboring the *AcF3H* gene (pGWB5-*AcF3H*) was transferred into *A. tumefaciens* AGL1 by electroporation. Fifty nanograms of the expression vector and 40 µL of *A. tumefaciens* AGL1 competent cells were mixed and transferred into an electroporation cuvette, and then the vector was transformed into *A. tumefaciens* AGL1 by electroporation at 1.8 kV (Bio-Rad, USA). After adding 500 µL of LB medium, the transformed cells were incubated at 30 °C and 150 rpm for 4 h. Thereafter, 50 µL of cell suspension was spread on LB medium supplemented with 25 µg/mL each of hygromycin and carbenicillin and incubated at 30 °C for 2 days. The presence of the expression vector in the positive clones was confirmed by PCR amplification and DNA sequencing.

2.5. *Agrobacterium*-mediated transformation of *Dendrobium* 5N white plants with the *AcF3H* gene

The *A. tumefaciens* strain AGL1 carrying the expression vector pGWB5-*AcF3H* was cultured at 30 °C in liquid LB medium containing 25 mg/L hygromycin, 25 mg/L carbenicillin, and 200 µM of acetosyringone. After 24 h of cultivation, 1 µL of cell suspension was transferred into fresh LB medium containing the same concentration of antibiotics and acetosyringone and then incubated at 30 °C until the optical density of the cells at the wavelength of 600 nm (OD_{600}) was 1.0. An amount of 2 g of PLBs (0.4–0.6 cm in diameter) was soaked in 20 mL of the *Agrobacterium* cell suspension for different incubation times (15, 30, and 45 min) and incubated at 25 °C in an incubator shaker at 100 rpm. Thereafter, they were blotted on sterile filter paper and placed on solid PLB multiplication medium containing 100 µM acetosyringone (Men et al., 2003). The PLBs were cocultivated with *Agrobacterium* at 28 °C for 3 days in the dark. After cocultivation, they were washed twice with 500 mg/L cefotaxime and placed on solid PLB multiplication medium supplemented with 30 mg/L hygromycin and 200 mg/L cefotaxime (Chai et al., 2002; Atichart et al., 2007).

2.6. Selection and confirmation of transgenic plants

Transformed PLBs were screened on modified solid VW medium supplemented with 15% (v/v) coconut water, 100 g/L PH, 200 mg/L cefotaxime, 30 mg/L hygromycin, and 6 g/L agar. After 2 months of cultivation, the hygromycin-resistant PLBs were transferred to modified VW medium

containing 15% (v/v) coconut water and 10 g/L agar without antibiotic supplementation. The genomic DNA of the transformed and nontransformed PLBs was extracted using the method of Quintanilla-Quintero et al. (2011) with minor modifications (without polyvinylpyrrolidone and dithiothreitol in the CTAB buffer). To confirm the insertion of a transgene, a 556-bp fragment of the 35S promoter and a 453-bp fragment of the *hpt* gene were amplified using PCR with sets of primers as follows: 35S-F (5'-AAGACGATCTACCCGAGCAA-3'), 35S-R (5'-CAACGATGGCCTTTCCTTTA-3') and *hpt*-F (5'-GATGTTGGCGACCTCGTATT-3'), *hpt*-R (5'-GAATTCAGCGAGAGCCTGAC-3'). The PCR amplification was performed for 35 cycles using the following profiles: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min.

2.7. Transient expression analysis of *Dendrobium* petals with the *AcF3H* gene using the agroinfiltration technique

The *A. tumefaciens* strain AGL1 carrying the expression vector pGWB5-*AcF3H* was grown for 24 h at 30 °C in LB medium containing 25 mg/L hygromycin and 25 mg/L carbenicillin. One milliliter of the cell suspension was transferred to 100 mL of LB medium containing the same concentration of antibiotics and grown overnight. Preparation of the cell suspension for infiltration was carried out using the method described by Yasmin and Debener (2010). The cells were harvested by centrifugation at 4500 rpm and 22 °C for 15 min. The resulting cell pellet was washed twice with sterile distilled water and then resuspended in an amount of sterile distilled water that resulted in an OD_{600} of 0.5. The petals of *Dendrobium* 5N white and *Dendrobium* Anna plants were infiltrated via a hole punctured at the base of the petal with the *Agrobacterium* suspension using a 1-mL needleless syringe (Schöb et al., 1997; Yasmin and Debener, 2010). Negative control petals were infiltrated with sterile distilled water. The infiltrated petals were kept on a petri dish with wet tissue paper and incubated at 25 °C under an 8/16-h (light/dark) photoperiod. Cyanidin content was measured from the petals from 0 to 5 days after agroinfiltration.

2.8. Cyanidin extraction and HPLC analysis

The cyanidin in the infiltrated petals and negative control petals was extracted using the method described by Zhang et al. (2004). The petals were ground using a mortar and pestle and dispersed in a 1:1 water/methanol solution containing 2 N HCl. The sample was sonicated for 20 min and filtered through a 0.2-µm nylon membrane filter. The filtrated solution was hydrolyzed at 100 °C for 60 min and cooled down at room temperature. The cyanidin content in the hydrolyzed sample was analyzed using HPLC as described by Zhang et al. (2004) with some modifications. Briefly, the sample was fractionated at 40 °C on a C18 reverse-phase column (Nova-Pak 60 Å, 4 µM, 3.9 × 300 mm, Waters) at a flow rate of 0.9 mL/min. Cyanidin was separated with 18% acetonitrile containing 0.4% trifluoroacetic acid and analyzed at a wavelength of 524 nm.

3. Results and discussion

3.1. Optimization of culture medium for PLB proliferation

PLBs of the *Dendrobium* 5N white plants were grown on modified VW medium supplemented with 15% (v/v) coconut water, 0.6% agar, and different concentrations of sucrose and PH. The results revealed that the culture medium supplemented with 100 g/L PH resulted in the highest growth as measured by fresh weight (12.22 ± 1.92 g) after 6 weeks of cultivation (Figure 3). PLBs grown on this culture medium exhibited good characteristics, i.e. healthy plantlets with green color. In contrast, PLBs grown on culture medium containing sucrose at 40 g/L exhibited lower growth with a yellowish to brownish color and unhealthy form (Figure 4). Generally, the optimal level of sucrose concentration in the culture medium for plant micropropagation varies depending on the genus and plant species. In this study, it seemed likely that additional sucrose in the modified VW medium did not enhance the growth of the *Dendrobium* PLBs. This may be because the modified VW medium contained a sufficient carbon source for PLB growth since it is composed of organic additives, such as coconut water and PH. It has been reported that coconut water and PH contain sucrose, fructose, and glucose as the major sugars, which can be used as carbon and energy sources for the growth of plant cells (Schwimmer et al., 1954; Yong et al., 2009). In nobile-type *Dendrobium* hybrids, sucrose at different concentrations (0, 10, 20, 40 g/L) had no effect on seed germination, but it did affect PLB development. A high concentration of sucrose (40 g/L) improved the development of PLBs with a yellowish color and rough surface form (Udomdee et al., 2014). In *Cymbidium* orchids, the proliferation of PLBs in a culture medium containing 30 g/L sucrose was greater than that in culture medium containing 10 g/L sucrose. In contrast, the proliferation of PLBs was inhibited when sucrose concentration in the culture medium was greater than 60 g/L. This may be due to the high osmotic stress caused by a high sugar concentration (Chin et al., 2007). In *Phalaenopsis* orchids, the optimum level of sucrose for PLB proliferation was found to be 40 g/L (Chai et al., 2002).

Several organic additives have been used to supplement the culture medium for promoting PLB proliferation, such as potato, banana, apple, and tomato homogenates. In *Phalaenopsis*, the formation of new PLBs was greater in culture medium supplemented with 100 g/L PH (Chai et al., 2002). In *Vanda* Kasem's Delight, culture medium supplemented with 20% (w/v) potato extract was found to

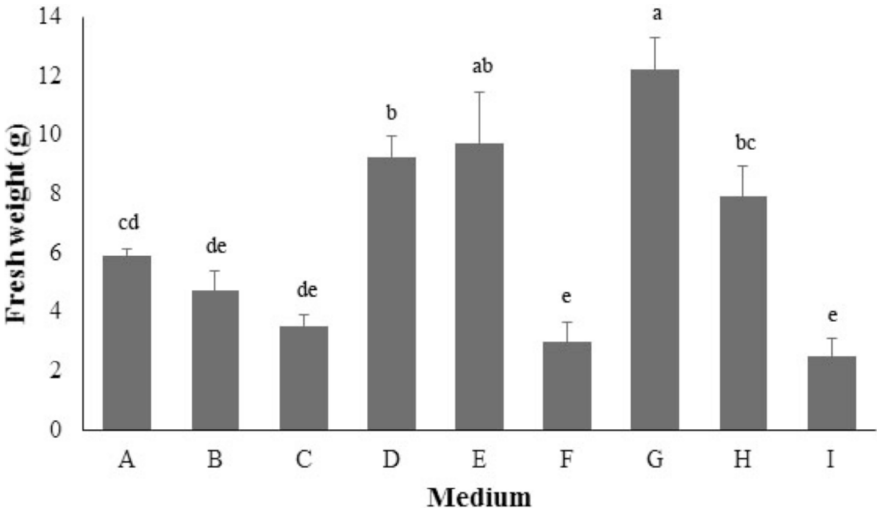

Figure 3. The fresh weight of PLBs of *Dendrobium* 5N white plants grown on solid VW medium with different concentrations of sucrose and potato homogenate after 6 weeks. Each culture medium contained the concentrations of sucrose:potato homogenate (g/L) as follows: A, 0:0; B, 20:0; C, 40:0; D, 0:50; E, 20:50; F, 40:50; G, 0:100; H, 20:100; I, 40:100. The data were analyzed using one-way ANOVA and the difference was analyzed using Duncan's multiple range test. Values with different letters indicate significant difference at $P < 0.05$. Bars represent the standard deviation (SD).

be the best condition for PLB proliferation as compared with other conditions (Gnasekaran et al., 2012).

3.2. Optimum concentration of hygromycin for screening the transgenic *Dendrobium* plants

The effect of hygromycin on the survival of PLBs was tested, and the results are summarized in Figure 5. At 30 mg/L hygromycin, no survival of PLBs was observed, suggesting that this concentration is suitable for screening the transgenic *Dendrobium* 5N white plants. At 30 mg/L hygromycin, nontransformed PLBs turned brown and completely died within 4 weeks (Figure 6). Our result is similar to that of Men et al. (2003), who reported that the optimum concentration of hygromycin for screening the transformed *Dendrobium nobile* was 30 mg/L. Screening of transgenic orchids using a low concentration of hygromycin has also been reported, e.g., Atichart et al. (2007) used hygromycin at a concentration of 25 mg/L for screening transgenic *Dendrobium secundum* plants. Chai et al. (2002) and Liau et al. (2003) reported screening of *Phalaenopsis* and *Oncidium* using hygromycin at a concentration of 3 and 5 mg/L, respectively. In addition, hygromycin at a concentration of 10 mg/L has also been used to screen transgenic *Vanda* and *Cattleya* plants (Shrestha et al., 2010; Zhang et al., 2010).

3.3. *Agrobacterium*-mediated transformation efficiency of the *AcF3H* gene into *Dendrobium* 5N white plants

PLBs of the *Dendrobium* 5N white plants (Figure 7A) were cocultivated with *Agrobacterium* harboring the expression vector pGWB5-*AcF3H*. Two months after screening the transgenic plants on culture medium containing hygromycin at 30 mg/L, the nontransformed PLBs turned brown and completely died (Figure 7B), while some parts of the transformed PLBs were still alive (Figure 7C). The hygromycin-resistant tissues were then transferred onto a new medium without antibiotic supplementation. Three months after cultivation, the transformed orchids generated leaves and roots (Figures 7D–7F). Successful *Agrobacterium*-mediated gene transformation into *Dendrobium* orchids has been infrequently reported (Men et al., 2003; Atichart et al., 2007; Phlaetita et al., 2015). The efficiency of *Agrobacterium*-mediated transformation depends upon several factors, such as the type of explants, strain and concentration of bacterial cells, type of inoculation medium, incubation and cocultivation time, the *Agrobacterium*-eliminating agent, and the method used to screen and regenerate the transgenic plants. In the present study, the plant tissues were incubated with the *Agrobacterium* suspension for different lengths of time, and the effect on the transformation efficiency was investigated, with the results summarized in the Table. After 1 month of transformation, there was no significant difference in transformation efficiency at different incubation times. This may be due to the presence of chimeric cells that are still resistant to hygromycin. However, stable transgenic plants were obtained after 2 months of transformation. The highest transformation efficiency (10.13%) was achieved using an incubation time of 15 min. It should be noted

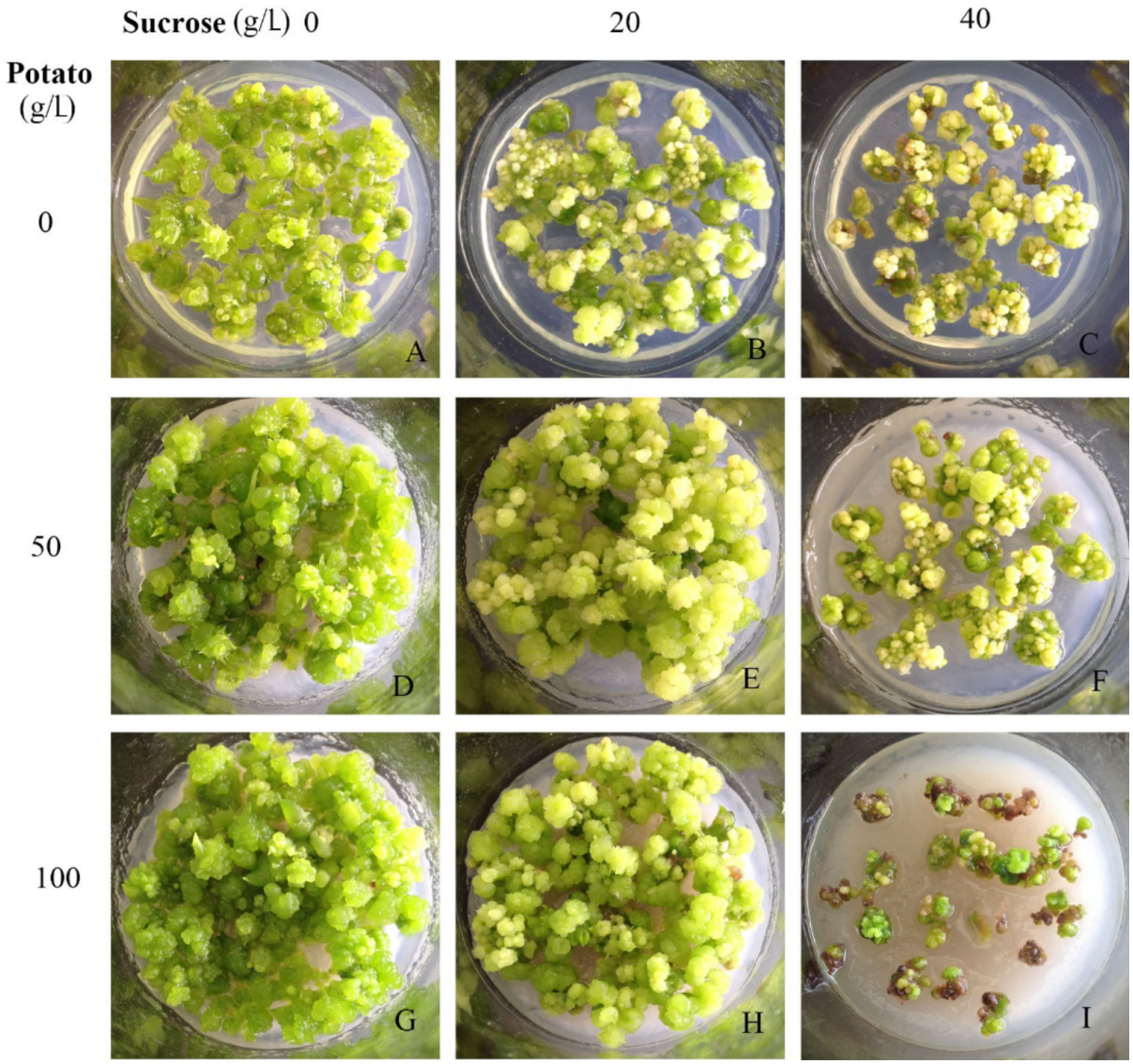

Figure 4. PLBs of *Dendrobium* 5N white plants grown on solid VW medium containing different concentrations of sucrose and potato homogenate. Each medium contained the concentrations of sucrose:potato homogenate (g/L) as follows: A, 0:0; B, 20:0; C, 40:0; D, 0:50; E, 20:50; F, 40:50; G, 0:100; H, 20:100; and I, 40:100.

from this study that longer incubation times (30 and 45 min) resulted in a reduction in transformation efficiency, similar to that reported in *Dendrobium nobile*. When the incubation period was increased from 30 to 60 min, the transformation efficiency decreased from 18% to 3%–4% (Men et al., 2003). In *Dendrobium* Formidible 'Ugusu', the highest transformation efficiency (12.2%) was achieved when PLBs were incubated with the bacterial suspension (OD_{600} = 0.1) for 30 min (Phlaetita et al., 2015). A longer incubation time for *Agrobacterium*-mediated gene transformation has also been reported in other orchids, such as *Vanda*, for which it was found that a 4-h incubation period gave the highest transformation efficiency. With incubation periods greater than 8 h, however, PLB necrosis was observed (Shrestha et al., 2010).

3.4. Selection and confirmation of transgenic orchids

Two months after screening on culture medium containing hygromycin, hygromycin-resistant PLBs were transferred to a culture medium without hygromycin. To verify the presence of the *AcF3H* gene in the transgenic *Dendrobium* plants, the *hpt* gene and 35S promoter were amplified using PCR with specific primers. This study found that PCR products of approximately 400 and 500 bp of the *hpt* gene and the 35S promoter, respectively, were amplified in all transgenic orchids (Figure 8), while no PCR product was found in nontransgenic plants, suggesting that the *AcF3H*

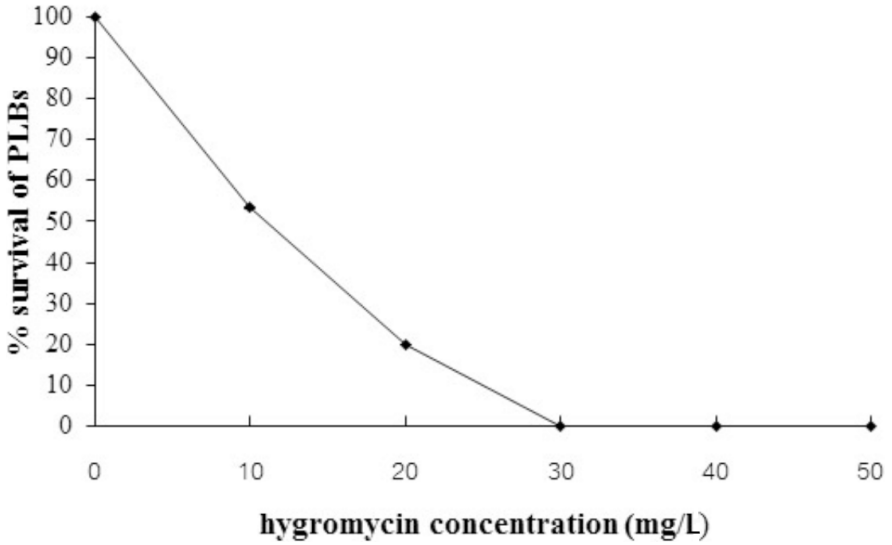

Figure 5. Percentage of survival of *Dendrobium* 5N white PLBs after 4 weeks of culture in a medium containing different concentrations of hygromycin.

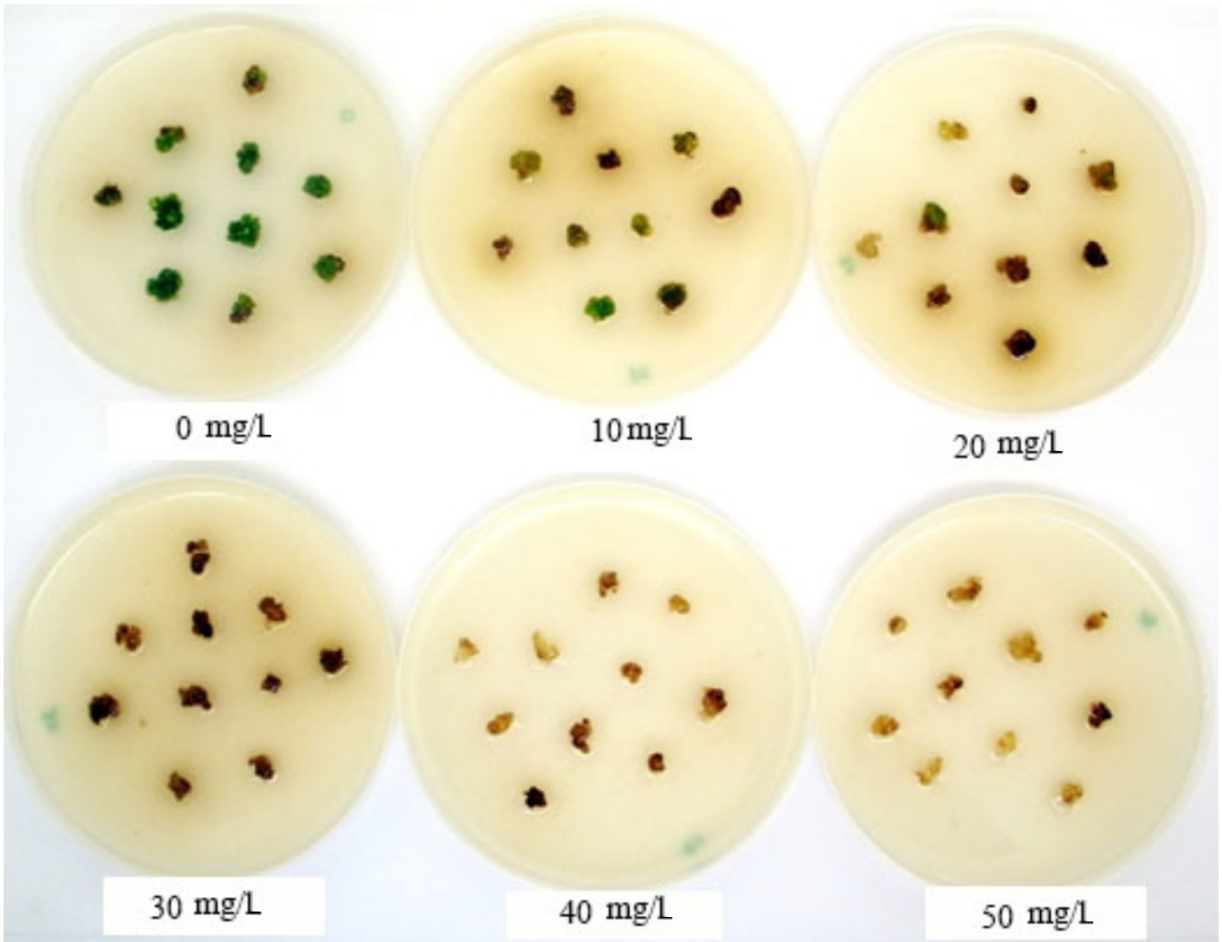

Figure 6. PLBs of *Dendrobium* 5N white plants grown on culture medium containing different concentrations of hygromycin (0, 10, 20, 30, 40, or 50 mg/L) for 1 month.

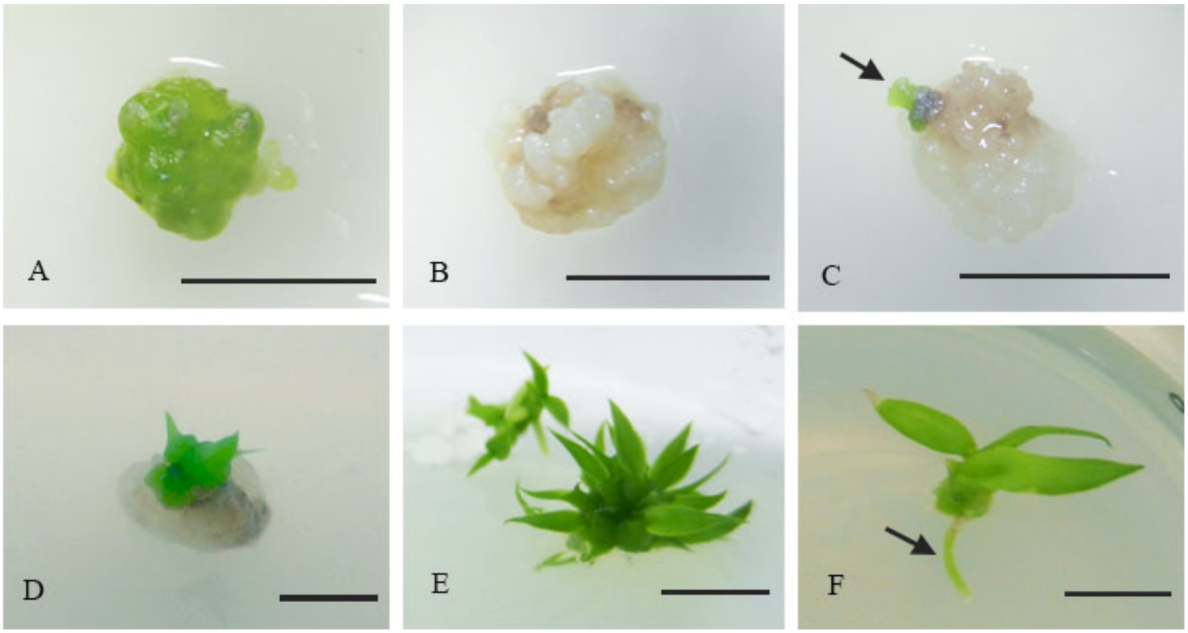

Figure 7. PLBs and plantlets of *Dendrobium* after *Agrobacterium*-mediated *F3H* gene transformation. A) PLBs before transformation. B) Nontransformed PLBs, dead after screening with hygromycin for 1 month. C) Hygromycin-resistant cell clusters (arrow) after 1 month of transformation on medium supplemented with 30 mg/L hygromycin. D) Transgenic plantlets 2 months after transformation. E) Transgenic plantlets 3 months after transformation. F) Transgenic plantlets 4 months after transformation; the roots were generated as indicated by the arrows (bars represent 5 mm).

Table. *Agrobacterium*-mediated gene transformation efficiency of the *AcF3H* gene into PLBs of *Dendrobium* 5N white orchid after transformation for 1 and 2 months.

Incubation time (min)	Number of PLBs per 2 g	One month after transformation		Two months after transformation	
		Number of *hyg*^{R} calli*	Transformation efficiency (%)	Number of *hyg*^{R} calli*	Transformation efficiency (%)
15	35.33 ± 1.53	6.00 ± 1.53	12.40 ± 4.80^a	3.5 ± 0.71	10.13 ± 1.84^a
30	37.67 ± 2.08	3.66 ± 2.08	9.76 ± 5.67^a	0.33 ± 0.58	0.93 ± 1.60^b
45	35.00 ± 3.46	2.33 ± 1.53	6.76 ± 4.75^a	0.33 ± 0.58	1.01 ± 1.75^b
Control	34.67 ± 1.53	0	0	0	0

The transformation efficiency was calculated as the percentage of hygromycin-resistant calli and presented as mean ± SD. The data were analyzed using one-way ANOVA and differences were determined using Duncan's multiple range test. Values with different letters indicate significant difference at $P < 0.05$. * represents number of hygromycin-resistant calli.

gene was integrated into the genome of the transgenic orchids.

3.5. Transient expression analysis of the *AcF3H* gene in the petals of *Dendrobium* orchid using agroinfiltration

Evaluations of transgene expression and stability in transgenic plants are laborious and time-consuming, particularly in transgenic orchids (at least 3 to 4 years). Therefore, a new technique for transient gene expression in plants that is simple, rapid, and reproducible is needed. Agroinfiltration is one of the most powerful techniques in plant biotechnology, especially transient gene expression analysis. It has been widely used to study gene expression in several plants, such as carnation (Zuker et al., 2002), rose (Yasmin and Debener, 2010), *Lactuca sativa* (Ren et al., 2011), strawberry (Jiang et al., 2013), and tobacco (Mahajan and Yadav, 2014; Meng et al., 2015; Song et al., 2016). In this study, agroinfiltration was employed for the transient expression analysis of the *AcF3H* gene

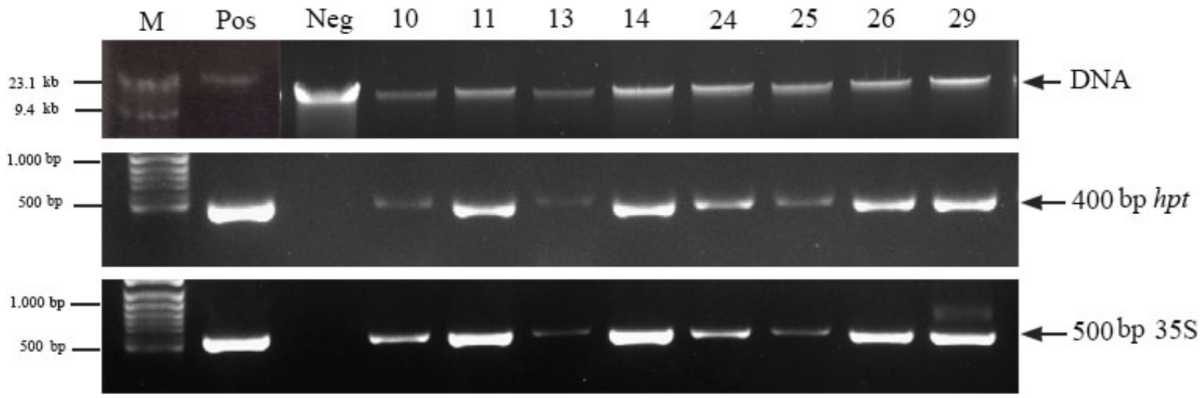

Figure 8. PCR amplification of the *hpt* gene and 35S promoter of transgenic orchids. M is DNA marker, Lane pos. is the PCR product of the positive control (pGWB5 vector), and Lane neg. is the nontransformed plant (negative control). Transformed orchids with pGWB5-*AcF3H* are indicated by Nos. 10, 11, 13, 14, 24, 25, 26, and 29. DNA was extracted from transformed and nontransformed orchids and used as the template for PCR amplification. The pGWB5 vector was used as the template for the positive control.

in petals of *Dendrobium* 5N white (white flowers) and *Dendrobium* Anna (purple flowers). The expression of *AcF3H* was regulated by the strong and constitutive CaMV 35S promoter. This promoter has been successfully used to express genes in several plants, such as maize and rose (Tanaka et al., 1998; Katsumoto et al., 2007). An *A. tumefaciens* suspension carrying the expression vector pGWB5-*AcF3H* was infiltrated into *Dendrobium* 5N white and *Dendrobium* Anna petals. Negative control petals were infiltrated with sterile distilled water. Cyanidin contents in orchid petals from 0 to 5 days after infiltration were measured by HPLC. No cyanidin was detected in *Dendrobium* 5N white petals after infiltration with the *AcF3H* gene. This may be due to the absence of F3'H activity in this orchid, similar to that reported in white *Torenia*, which lacks F3'H and F3'5'H activity. Only pelargonidin accumulated in white *Torenia* after transformation with a foreign gentian *F3H* gene, and no cyanidin or delphinidin was observed (Nishihara et al., 2014). In *Dendrobium* Anna petals, the cyanidin content was slightly increased to 16.17 μg/g FW on day 2 after infiltration (a 6% increase from day 0) (Figure 9), suggesting that the *AcF3H* gene was transiently expressed in the *Dendrobium* Anna petals after infiltration. In contrast, the cyanidin content in the negative control decreased to 44.09 μg/g FW on day 1 after infiltration (a 15.87% decline from day 0). This may have been caused by the degradation of cyanidin. Normally, the petals of *Dendrobium* Anna flowers contain cyanidin-based anthocyanin, as analyzed by HPLC, indicating the presence of F3'H activity in this orchid. Therefore, the transformation of the foreign *AcF3H* gene into *Dendrobium* Anna petals caused an increase in the accumulation of cyanidin in the petals. F3H is involved in the formation of dihydroflavonol, which is an intermediate substance in flavonol, anthocyanidin, and proanthocyanidin biosynthesis (Prescott and John, 1996). Flavonol glycosides can also be detected and identified as the flavonol aglycones kaempferol, quercetin, and myricetin and the methylated derivatives isorhamnetin and syringetin (Kuehnle et al., 1997). Thus, the presence of other flavonoid compounds should also be determined in *Dendrobium* 5N white and *Dendrobium* Anna petals after infiltration.

It was reported in a previous study that suppression of the *F3H* gene in carnations (orange flowers) resulted in various flower colors, ranging from partial to complete loss of the original color (Zuker et al., 2002). Infiltration of *Agrobacterium* harboring the *F3H* RNAi vector into strawberry fruits resulted in the *F3H* gene being downregulated by approximately 70% compared with the control. The levels of anthocyanins and flavonols in the *F3H*i fruit, including quercetin glucuronide, kaempferol glucuronide, pelargonidin 3-glucoside, pelargonidin 3,5-diglucoside, and pelargonidin 3-rutinoside, were much lower than those of the control fruit (Jiang et al., 2013). In addition, overexpression of the *F3H* gene in other plants has also been investigated. Overexpression of the *L. chinense F3H* resulted in an increase in the flavonoid (flavan-3-ol) content and an ability to tolerate drought stress in transgenic tobacco (Song et al., 2016). Mahajan and Yadav (2014) reported the overexpression of the *C. sinensis F3H* gene in tobacco and found that the flavan-3-ol content in the transgenic plants was remarkably increased compared with that of nontransgenic plants. In addition, transgenic plants also exhibited a greater tolerance to salt stress and resistance to *Alternaria solani* infection through improved antioxidant activity and enhanced pectin methyl esterification. Meng et al. (2015) reported the overexpression of the tomato *F3H*-like protein gene in tobacco and found that transgenic plants accumulated

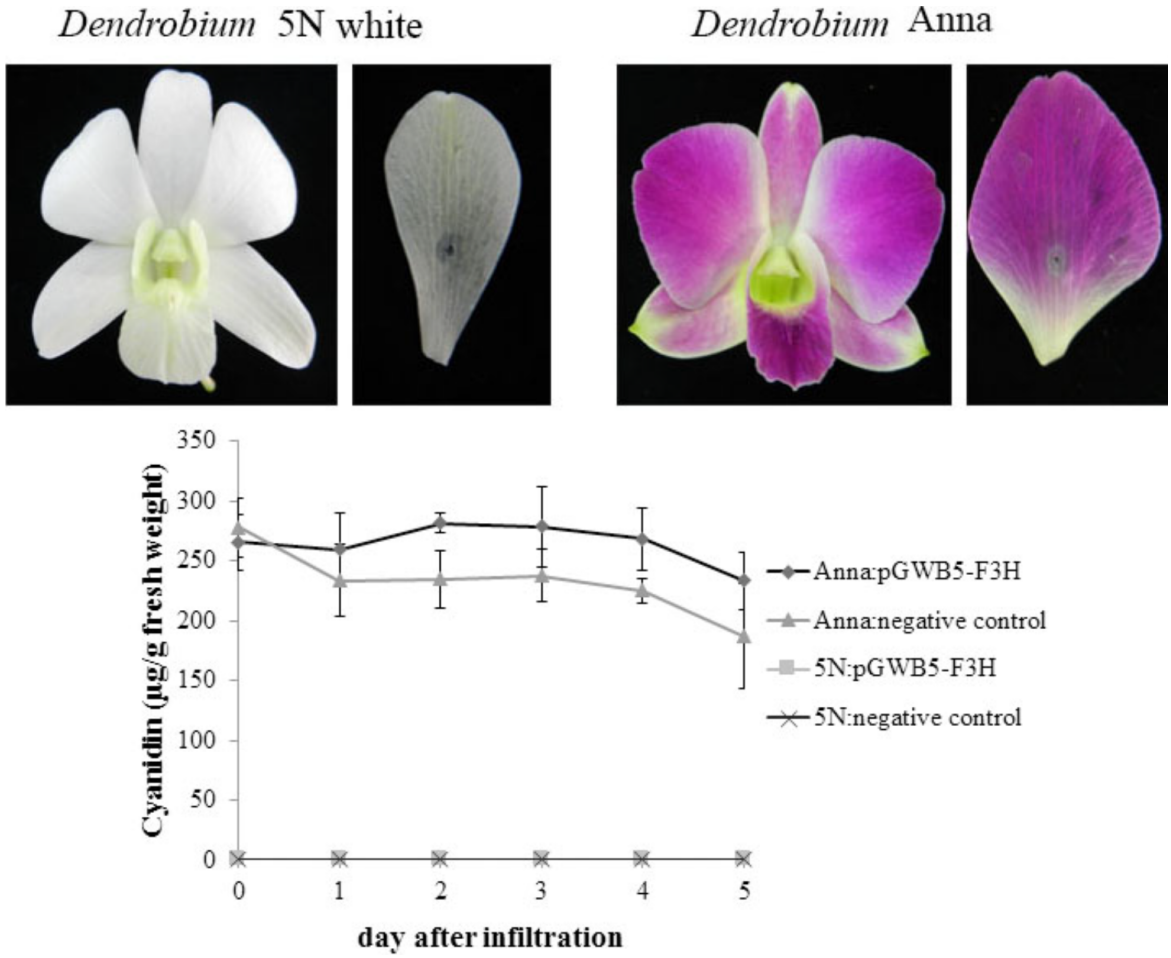

Figure 9. Cyanidin content in the *Dendrobium* 5N white and *Dendrobium* Anna petals after agroinfiltration with *A. tumefaciens* harboring a pGWB5-*AcF3H* vector. Water was injected into the petals as the negative control. The *Dendrobium* 5N white and *Dendrobium* Anna petals are shown in the upper panel. Levels of cyanidin content in the orchid petals are shown in the lower panel. Bars represent the standard deviation (SD).

high amounts of flavonoids and had increased tolerance to chilling stress compared with nontransgenic plants.

In summary, modified VW medium supplemented with 100 g/L PH was found to be the best for PLB proliferation of *Dendrobium* 5N white orchid plants. Plant expression vector pGWB5-*AcF3H* harboring the *Ascocenda F3H* gene was successfully constructed using the Gateway cloning system. This expression vector was transformed into PLBs of *Dendrobium* 5N white orchid plants using *Agrobacterium*-mediated transformation. The highest transformation efficiency (10.13%) was achieved when PLBs were cocultivated with *A. tumefaciens* strain AGL1 harboring the pGWB5-*AcF3H* for 15 min. Based on the PCR analysis, we propose that the *AcF3H* gene was integrated into the genome of transgenic *Dendrobium* 5N white orchid plants. We also demonstrated in this study that agroinfiltration could be used to evaluate the transient expression of the *AcF3H* gene in *Dendrobium* 5N white and *Dendrobium* Anna petals. Since F3H is involved in the biosynthesis of other flavonols and anthocyanidins, other flavonoid compounds should be analyzed in these orchids after agroinfiltration. The knowledge obtained in this study, particularly on the construction of the plant expression vector using the Gateway cloning system and the *Agrobacterium*-mediated gene transformation procedure, will be useful for further study on the modification of flower color in orchids, as well as other commercially important ornamental plants.

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References

Angers-Loustau A, Rainy J, Wartiovaara K (2007). PlasmaDNA: a free, cross-platform plasmid manipulation program for molecular biology laboratories. BMC Mol Biol 8: 77-81.

Atichart P, Bunnag S, Theerakulpisut P (2007). *Agrobacterium*-mediated transformation of *Dendrobium secundum* (Bl.) Lindl with antisense ACC oxidase. Asian Journal of Plant Sciences 6: 1065-1071.

Chai ML, Xu CJ, Senthil KK, Kim JY, Kim DH (2002). Stable transformation of protocorm-like bodies in *Phalaenopsis* orchid mediated by *Agrobacterium tumefaciens*. Sci Hortic-Amsterdam 96: 213-224.

Chin DP, Mishiba K, Mii M (2007). *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cymbidium*. Plant Cell Rep 26: 735-743.

Gnasekaran P, Poobathy R, Mahmood M, Samian MR, Subramaniam S (2012). Effect of complex organic additives on improving the growth of PLBs of *Vanda* Kasem's Delight. Australian Journal of Crop Science 6: 1245-1248.

Holton T, Cornish E (1995). Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7: 1071-1083.

Jiang F, Wang J, Jia H, Jia W, Wang H, Xiao M (2013). RNAi-mediated silencing of the flavanone 3-hydroxylase gene and its effect on flavonoid biosynthesis in strawberry fruit. J Plant Growth Regul 32: 182-190.

Katsumoto Y, Fukuchi-Mizutani M, Fukui Y, Brugliera F, Holton TA, Karan M, Nakamura N, Yonekura-Sakakibara K, Togami J, Pigeaire A et al. (2007). Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. Plant Cell Physiol 48: 1589-1600.

Khumkarjorn N, Thanonkeo S, Yamada M, Thanonkeo P (2016). Cloning and expression analysis of a flavanone 3-hydroxylase gene in *Ascocenda* orchid. J Plant Biochem Biot 26: 179-190.

Kuehnle AR, Lewis DH, Markham KR, Mitchell KA, Davies KM, Jordan BR (1997). Floral flavonoids and pH in *Dendrobium* orchid species and hybrids. Euphytica 95: 187-194.

Kumar A, Singh B, Singh K (2015). Functional characterization of flavanone 3-hydroxylase gene from *Phyllanthus emblica* (L.). J Plant Biochem Biot 24: 453-460.

Liau CH, You SJ, Prasad V, Hsiao HH, Lu JC, Yang NS, Chan MT (2003). *Agrobacterium tumefaciens*-mediated transformation of an *Oncidium* orchid. Plant Cell Rep 21: 993-998.

Mahajan M, Yadav SK (2014). Overexpression of a tea flavanone 3-hydroxylase gene confers tolerance to salt stress and *Alternaria solani* in transgenic tobacco. Plant Mol Biol 85: 551-573.

Men S, Ming X, Liu R, Wei C, Li Y (2003). *Agrobacterium*-mediated genetic transformation of a *Dendrobium* orchid. Plant Cell Tiss Org 75: 63-71.

Meng C, Zhang S, Deng YS, Wang GD, Kong FY (2015). Overexpression of a tomato flavanone 3-hydroxylase-like protein gene improves chilling tolerance in tobacco. Plant Physiol Bioch 96: 388-400.

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T (2007). Development of series of Gateway binary vector, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng 104: 34-41.

Nishihara M, Yamada E, Saito M, Fujita K, Takahashi H, Nakatsuka T (2014). Molecular characterization of mutation in white-flowered torenia plants. BMC Plant Biol 14: 86-97.

Phlaetita W, Chin DP, Tokuhara K, Nakamura I, Mii M (2015). *Agrobacterium*-mediated transformation of protocorm-like bodies in *Dendrobium* Formidible 'Ugusu'. Plant Biotechnol 32: 225-231.

Prescott AG, John P (1996). Dioxygenases: molecular structure and role in plant metabolism. Annu Rev Plant Phys 47: 245-271.

Quintanilla-Quintero S, Ortiz P, Bernal J, Gomez A (2011). Phylogenetic relationships among genera of the subtribe Oncidiinae (Epidendroideae: Orchidaceae) and a new genus: *Santanderella*. Phytologia 93: 388-406.

Ren W, Zhao L, Zhang L, Wang Y, Cui L, Tang Y, Sun X, Tang K (2011). Molecular cloning and characterization of 4-hydroxyphenylpyruvate dioxygenase gene from *Lactuca sativa*. J Plant Physiol 168: 1076-1083.

Saiprasad GVS, Raghuveer P, Khetarpal S, Chandra R (2004). Effect of various polyamines on production of protocorm-like bodies in orchid-*Dendrobium* 'Sonia'. Sci Hortic-Amsterdam 100: 161-168.

Schöb H, Kunz C, Meins F Jr (1997). Silencing of transgenes introduced into leaves by agroinfiltration: a simple, rapid method for investigating sequence requirements for gene silencing. Mol Genet Genomics 256: 581-585.

Schwimmer S, Bevenue A, Weston WJ, Potter AL (1954). Potato composition, survey of major and minor sugar and starch components of white potato. J Agr Food Chem 2: 1284-1290.

Shrestha BR, Chin DP, Tokuhara K, Mii M (2010). *Agrobacterium*-mediated transformation of *Vanda* using protocorm-like bodies. Asia-Pac J Mol Biol 18: 225-228.

Song X, Diao J, Ji J, Wang G, Guan C, Jin C, Wang Y (2016). Molecular cloning and identification of a flavanone 3-hydroxylase gene from *Lycium chinense*, and its overexpression enhances drought stress in tobacco. Plant Physiol Bioch 98: 89-100.

Tanaka Y, Brugliera F, Chandler S (2009). Recent progress of flower colour modification by biotechnology. Int J Mol Sci 10: 5350-5369.

Tanaka Y, Sasaki N, Ohmiya A (2008). Biosynthesis of plant pigment: anthocyanins, betalains and carotenoids. Plant J 54: 733-749.

Tanaka Y, Tsuda S, Kusumi T (1998). Metabolic engineering to modify flower color. Plant Cell Physiol 39: 1119-1126.

Tu Y, Liu F, Guo D, Fan L, Zhu Z, Xue Y, Gao Y, Guo M (2016). Molecular characterization of flavanone 3-hydroxylase gene and flavonoid accumulation in two chemotyped safflower lines in response to methyl jasmonate stimulation. BMC Plant Biol 16: 132.

Udomdee W, Wen P, Lee C, Chin S, Chen F (2014). Effect of sucrose concentration and seed maturity on *in vitro* germination of *Dendrobium nobile* hybrids. Plant Growth Regul 72: 249-255.

Vacin EF, Went FW (1949). Some pH changes in nutrient solutions. Bot Gaz 110: 605-613.

Xiong S, Tian N, Long J, Chen Y, Qin Y, Feng J, Xiao W, Liu S (2016). Molecular cloning and characterization of a flavanone 3-hydroxylase gene from *Artemisia annua* L. Plant Physiol Bioch 105: 29-36.

Yasmin A, Debener T (2010). Transient gene expression in rose petal via *Agrobacterium* infiltration. Plant Cell Tiss Org 102: 245-250.

Yong JWH, Ge L, Ng YF, Tan SN (2009). The chemical composition and biological properties of coconut (*Cocos nucifera* L.) water. Molecules 14: 5144-5164.

Zhang L, Chin DP, Mii M (2010). *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cattleya*. Plant Cell Tiss Org 103: 41-47.

Zhang Z, Kou X, Fugal K, McLaughlin J (2004). Comparison of HPLC methods for determination of anthocyanins and anthocyanidins in bilberry extracts. J Agr Food Chem 52: 688-691.

Zuker A, Tzfira T, Ben-Meir H, Ovadis M, Shklarman E, Itzhaki H, Forkmann G, Martens S, Neta-Sharir I, Weiss D et al. (2002). Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. Mol Breeding 9: 33-41.