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
Thus, the Gateway cloning system may be an alternative and reliable alternative approach (Nakagawa et al., 2007). Transformation has been developed and provides a fast series of Gateway binary vectors (pGWBs) for plant construction of expression vectors is very complicated, and have many restriction enzyme recognition sites, the vector. Since most plant expression vectors are large, 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 3² 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) attB1 and attB2 were added to the AcF3H gene by PCR using PrimeSTAR® HS DNA polymerase (Takara, Japan). The primers used were ATT-F3H-F: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAGCTAAAATCTCATTTA-3' (italic letters represent the attB1 site) and ATT-F3H-R: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAGCTAAAATCTCATTTA-3' (italic letters represent the attB2 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 attB-flanked F3H gene was introduced into the attP-containing donor vector, pDNOR201, by a BP reaction to generate an attL-containing entry clone, pDONR201-AcF3H. The BP reaction mixture contained 1 µL of attB-PCR product (~15–150 ng), 0.5 µL

443
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 attL-containing AcF3H gene from the entry clone was transferred to the attR-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 fluorescent 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 sequencing.

**Figure 1.** Construction of plant expression plasmid pGWB5-AcF3H using the Gateway cloning system (Invitrogen, USA). AcF3H, Asascenda flavanone 3-hydroxylase; att, recombination site; gfp, green fluorescent protein; ccdB, control of cell death B gene; PCR, polymerase chain reaction.
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'), hpt-F (5'-GATGTTGGCCGACCTCTGATT-3'), hpt-R (5'-GAATTCAAGGAGGCTGTGAC-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 Yasin 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; Yasin 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
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 Liu 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
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 (OD600 = 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.
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
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). Flavanol 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 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...
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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