

**Establishment of transformation protocol and
production of transgenic plants expressing blue gene in
*Dendrobium***

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**Establishment of transformation protocol and
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*Dendrobium***

Dendrobium における形質転換系の確立と青色遺
伝子を発現する形質転換体の作出

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Chapter 1
General Introduction

Chapter 1:

General Introduction

Introduction

Background of this study

The aim of this study was to determine the best and most useful procedure for the production of novel *Dendrobium*, using sufficient genetic manipulation.

The genus *Dendrobium* is one of the largest genera in the family Orchidaceae, comprising some 1,200 individual species, growing in a wide range of climates from hot, wet lowlands to high-altitude, colder mountainous areas. Dendrobiums are divided into several groups based on their growing conditions. They are distributed from the foothills of the Himalayas through Southeast Asia to Japan, Australia, Tasmania, and the Pacific Islands (Kamemoto et al. 1999) growing as epiphytes or lithophytes. Many species of this genus are important as popular orchids, grown as potted plants and cut flowers for commercial production. In 2005, orchids comprised 8% of the worldwide floriculture trade (Martin and Madassery 2005). Their free flowering and long vase life of two to three weeks are important characteristics that make orchids ideally suited for the cut-flower trade. Thailand is the world's largest exporter of *Dendrobium* and other tropical orchids. The cut flower export business is estimated to be worth over US 70 million (Lekawatana, 2010). Recently world production of potted *Dendrobium* plants has increased, with production on a large scale in many countries including China, Taiwan, Thailand, The Philippines, The United States of America, Japan and

Germany (Puchooa D, 2004). Potted *Dendrobiums* of wide color varieties, shapes, and long vase life are the fourth ranked flower and nursery crop in the USA, with sales value increased dramatically to 12% of total potted plants (Kuehnle et al. 2003). *Dendrobium nobile* is an orchid used for ornamental purposes. It is one of the orchids commonly available in flower shops, stores and garden centers, easy to cultivate with reliable flowering of many big, beautiful blossoms that keep fresh for a long time. In Japan, this species has been developed into elite cultivars, with numerous colors and shapes by Yamamoto *Dendrobiums*, Japan. *Dendrobium* Formidible has a character like *D. nobile* but showed hair on stem. It blooms large sized white flowers in early and mid summer but flower color is limited.

Breeding of *Dendrobium*

Numerous characters in *Dendrobium* are unique, frequent flowering and long vase life which leading to high demand in orchids market. Also, *Dendrobium* could be used as indoor and outdoor pot plants and cut flowers. However, diseases, new color and longevity are important strategies from the point for development to forward the high demand in the future.

Viral diseases can infect *Dendrobium* during cultivation, including Cymbidium mosaic virus (CyMV) and Odontoglossum ring spot virus (ORSV). These are considered the most prevalent and economically deleterious, and blight numerous commercially grown important orchid genera. Infection by CyMV can cause blossom brown necrotic streaks, reducing plant vigor and flower quality, thus affecting economic value (Hu et al. 1993). The virus is most commonly spread through mechanical transmission during propagation or flower harvest; it can be carried from plant to plant by shears, knives, or direct contact of plants (Ishii, 1968). From these reasons, the vigor plant of *Dendrobium* needs to produce a free disease and could be controlled from the sprout through tissue culture and molecular method.

Flower color displays on *Dendrobium* depending on accumulation of secondary compounds, including flavonoids and carotenoids. Anthocyanins are colored flavonoids and displayed predominantly purple, lavender and pink flowers due to cyanidin and peonidin accumulation (Rasika et al. 2013). Carotenoids are natural pigments that impart yellow, red, or orange colors to flowers (Park et al. 2015). Also more than one color can appear on different parts of the flower that could be developed through intraspecific and interspecific hybridizations. However, blue delphinidin is absent in *Dendrobium* that an impossible to produce the blue flower color by the conventional breeding method. Recently, the novel colored like the blue flower is a new target to produce the novel flower character.

The cut flower of *Dendrobium* is high demand and one of important species of orchids in the flower market. However, its problem showed like any cut flower, their face vase-life-related problem such as excessive water loss, decline in respiring substrate and sensitively to exogenous or endogenous ethylene that hastens senescence and wilting of the flowers (Hew 1994). This problem has more effect when exported them to far countries because most of produced countries are located in Asia such as Thailand, Singapore to Europe or America. For resolving, many studies focused on the effect and mechanism of ethylene inhibitors and sugars (Chandran et al. 2006). Chemical treatment and plant breeding were utilized widely to improve the longevity of cut flowers (Yamada et al. 2003). Therefore, molecular method such as RNAi method involving ethylene pathway (Gupta et al. 2013) is an essential target for improving long vase-life cut *Dendrobium*.

Above the situation, research work in many tropical and sub-tropical countries is concentrated on the continuous breeding of plants with desirable traits to produce high value export products. Maintaining the high quality of *Dendrobium* orchids is a new challenge, and has become most important in the flower market. Orchid breeders have tried to produce new cultivars through conventional sexual breeding. However, some traits such as the blue color

are difficult to generate because of the lack of genetic variability in the germplasm. New strains also have to be strong and resistant to viruses to reduce losses from diseases. For creating new varieties with such new traits, plant biotechnology (genetic manipulation) has to utilize all available protocols to create new varieties.

Genetic transformation of *Dendrobium*

Three steps are important and necessary for feasible plant genetic engineering: 1) plant tissue must be able to regenerate after introducing the target gene, 2) the gene transfer method and gene expression vectors must be compatible with the plant, and 3) the selection of shoots arising from the target tissue and identification of the transformation. In orchids, seeds, protocorms, protocorm-like bodies (PLBs), and meristematic tissues such as excised shoot tips and lateral buds can be used as materials for the transformation. Mainly, two methods have been reported to introduce the foreign genes into plants, particle bombardment (Chia et al. 1994; Kuehnle and Sugii 1992; Suwanaketchanatit et al. 2007; Tee et al. 2003; Yu et al. 2001), and *Agrobacterium*-mediated transformation (Julkifle et al. 2012; Men et al. 2003; Pimda & Bunnag 2010; Yu et al. 2001). Particle bombardment requires the necessary equipment, while *Agrobacterium*-mediated transformation needs no extra equipment and is an easy and convenient method.

The establishment of an efficient transformation system of *Dendrobium* is necessary for genetic improvement to produce ornamental and marketable qualities, such as novel blue flower color, virus resistance, and stress tolerance.

***Agrobacterium*-mediated gene transfer**

Over the past decade, the *Agrobacterium*-mediated gene transformation method has been widely used for molecular breeding in various plant species. *Agrobacterium* is a gram-negative soil-borne pathogen, well-known for its ability to transfer DNA between itself and

plants. It harbors large plasmids called Ti-plasmid, which induce crown galls in the host plant cells and integrate randomly into the plant genomes. *Agrobacterium*-mediated transformation takes advantage of this natural mechanism by incorporating the T-DNA into the plant cells at the wounded sites. However, monocotyledonous plants are rarely natural hosts for *Agrobacterium* and difficult to transform. To solve the problem, various studies have been conducted by examine several factors that affect transformation efficiency including explants, *Agrobacterium* strain and plasmid, pretreatment, inoculation and cultivation conditions, and *Agrobacterium* density (Cheng et al. 2004; Sood et al. 2011). The optimal factors must be determined to produce transgenic varieties that can regenerate, without using chimeric or escape plants.

Color pigmentation

Flower color is one of the most attractive characteristics in ornamental plants, contributing major value in the floricultural market. In nature, three classes of pigments; i.e., flavonoids, carotenoids, and betalains, determine the coloration of flowers. Kuenhle et al. (1997) analyzed floral flavonoids in *Dendrobium*. Anthocyanidins, water-soluble flavonoid pigments were detected in 28 *Dendrobium* species and hybrids selected for analysis, based on color and suitability for cut flower breeding. Flowers colored pink, red, maroon, orange, bronze, and brown following the standard of The Royal Horticultural Society (RHS) contained anthocyanins based on cyanidin, with peonidin occurring as a minor pigment. The flower of *D. gouldii*, *D. bigibbum*, and *D. kultana* contained anthocyanins based on cyanidin. Pink flowers were classified as red or red purple, and included pelargonidin glycosides. Unfortunately, *Dendrobium* lacks blue flowers due to the absence of delphinidin-based anthocyanins. Delphinidin is one of the key anthocyanins, which produces the blue color in flowers; it is not available for synthesis by itself. Flavonoid-3', 5'-hydroxylase (F3'5'H) leads to the formation of dihydromyricetin (DHM) and subsequently to the production of

delphinidin-based pigments. Blue flowers have been successfully produced in several important ornamental plants including rose, chrysanthemum and carnation (Brugliera et al. 2013; Fukui et al. 2003; Katsumoto et al. 2007; Noda et al. 2013). Therefore, production of blue dendrobiums was targeted for *D.nobile* and *D. Formidible* in this experiment.

Aim of the study and outline of the thesis

Highly efficient genetic transformation systems were established by *Agrobacterium*-mediated transformation for the introduction of flavonoid-3', 5'-hydroxylase (F3'5'H) gene and related anthocyanin biosynthesis genes into *D. nobile* and *D. Formidible* using two types of plant materials. For *D. nobile*, protocorms were infected with *Agrobacterium* with different inoculation times and bacteria concentrations in Chapter 2, and PLBs for *Dendrobium Formidible* as target material in Chapter 3. From the optimum transformation system, F3'5'H gene isolated from *Commelina communis* was introduced to *D. nobile*, with different genetic backgrounds of seedlings (protocorms) derived from the cross between elite cultivars in Chapter 4. Finally, general conclusions, discussions and recommendations for further experiments are presented in Chapter 5, thereby showing the usefulness of molecular breeding or engineering of *Dendrobium* to create new cultivars with specific and novel features.

Chapter 2

**High efficiency *Agrobacterium*-mediated transformation of
Dendrobium orchid using protocorms as a target material**

Chapter 2: High efficiency *Agrobacterium*-mediated transformation of *Dendrobium* orchid using protocorms as a target material

Introduction

Dendrobium is one of the largest genera in the family Orchidaceae and includes the most popular orchids grown for commercial production of cut flowers and pot plants. The commonly cultivated species include *D. phalaenopsis*, *D. gouldii* and *D. nobile*. Recently, cultivars with novel flower colors, shapes and disease resistance have been developed through intraspecific and interspecific hybridizations (Kamemoto et al. 1999). However, conventional breeding in this genus by means of sexual hybridization is restricted due to long generation time, generally 3 years from seed sowing to flowering and lack of useable genetic variability (Kuehnle and Sugii 1992). Therefore, genetic engineering remains an important tool for producing *Dendrobium* with desirable traits such as blue flowers, disease resistance and long flower life.

In the last 15 years, genetic transformation of orchids has been reported using *Agrobacterium*-mediated method by targeting protocorm-like bodies (PLBs) as material for *Phalaenopsis* (Belarmino and Mii 2000; Chai et al. 2002; Sjahril and Mii 2006), *Cymbidium* (Chen et al. 2002; Chin et al. 2007), *Vanda* (Shrestha et al. 2007), *Cattleya* (Zhang et al. 2010), *Oncidium* (Liau et al. 2003) and *Dendrobium* (Nan et al. 1998; Yu et al. 2001; Men et al. 2003b). It is natural to use PLBs as the target for genetic transformation because most cultivars of these commercially important orchids are vegetatively propagated by tissue culture, and hence targeting the cultivars with superior traits is usually needed. In transgenic plants, however, expression of foreign genes is sometimes affected greatly by the genetic background of the target plants (Cogan et al. 2001). Therefore, it is necessary to introduce such genes into the plants with diverse genotypes to evaluate the usefulness or effectiveness of the target genes for transformation. Based on such idea, Mishiba et al. (2005) have already

reported successful *Agrobacterium*-mediated transformation with rapid selection of transgenic plants in *Phalaenopsis* by targeting protocorms at an early stage after germination. In *D. nobile*, however, protocorms have not been used as target material although PLBs have successfully been transformed by using both biolistic and *Agrobacterium*-mediated methods (Men et al. 2003a; 2003b)

In the present study, we tried to develop a simple and efficient procedure for *Agrobacterium*-mediated transformation by using the protocorms obtained from the cross between two cultivars of *D. nobile*-type hybrid as target material.

Materials and Methods

Plant material

Seeds of *Dendrobium nobile*-type hybrid obtained from a cross between *D. Flower Palace* ‘Cinderella’ × *D. Santa Isabel* ‘True Love’ were kindly provided by Mr. Nobuyuki Asai, Asai Daikeien Co. Ltd. To produce protocorms, seeds were surface-sterilized by agitating for 10 min in a sodium hypochlorite solution containing 1% (v/v) active chlorine, and then rinsed three times with sterile distilled water. Sterilized seeds were cultured in 35 ml of liquid New Dogashima (ND) medium (Tokuhara and Mii 1993) containing 10 g L⁻¹ maltose without adding any plant growth regulators (pH 5.4) in 100 ml flask and agitated at 80 rpm. All of the seeds were incubated at 25°C under constant illumination (33 μmol m⁻² s⁻¹) with cool-white-fluorescent lamps (National FL30SN, Osaka, Japan).

Plasmid vector and bacterial strain

Agrobacterium tumefaciens strain EHA101 (Hood et al. 1986), which harboring with binary vector pIG121Hm (Ohta et al. 1990), which contains a hygromycin phosphotransferase gene (*hpt*) and an intron *GUS* gene, both under the control of a 35S cauliflower mosaic virus promoter, and a neomycin phosphotransferase II gene (*nptII*) under the control of a nopaline synthase promoter in the T-DNA region was used.

Inoculation and co-cultivation with *Agrobacterium*

Agrobacterium was grown overnight at 28°C in LB liquid medium containing 50 mg L⁻¹ hygromycin (Hygromycin B; Wako Pure Chemical Industries, Osaka, Japan), 50 mg L⁻¹ kanamycin (Kanamycin sulfate; Wako Pure Chemical Industries, Osaka, Japan) and 25 mg L⁻¹ chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA). The bacterial suspension was diluted to final density of OD₆₀₀≈1.0 in inoculation medium, which was liquid ND medium containing 100 µM acetosyringone (AS) (3', 5'-dimethoxy-4'-hydroxy-acetophenone; Sigma-Aldrich, St. Louis, MO, USA). For inoculation, 21 day-old protocorms (Mishiba et al. 2005), obtained as described above were inoculated 3.5 ml (1/10) or 700 µl (1/50) of *Agrobacterium* suspension culture for either 30 or 300 min with mild agitation at 80 rpm on rotary shaker. The protocorms were then collected with a 40 µm nylon mesh placed on a funnel and immediately transferred with the nylon mesh onto a 0.25% (w/v) gellan gum (Wako Pure Chemical Industries)-solidified ND medium supplemented with 100 µM AS and co-cultivated for 3 days.

Elimination of bacteria, selection and plant regeneration

For selection and elimination of bacteria, protocorms were washed with liquid ND medium and placed onto 40 µm nylon mesh, which was overlaid on 0.25% (w/v) gellan gum-solidified ND medium containing 30 mg L⁻¹ hygromycin and 20 mg L⁻¹ meropenem trihydrate (Meropen; Dainippon Sumitomo Pharma, Osaka, Japan <http://www.ds-pharma.co.jp>) a β-lactam antibiotic (Ogawa and Mii 2004, 2007), for primary selection of transformants. The protocorms were subcultured every 3 weeks to fresh medium of the same composition. After selection for 3 months, protocorms showing green coloration were cut transversely into two pieces and placed onto ND medium without hygromycin but with meropenem (recovery medium) (Mishiba et al. 2005) and cultured for 1 month. Then, newly proliferated PLBs from protocorms on this medium were selected and returned to selection medium containing 30 mg L⁻¹ hygromycin and 10 mg L⁻¹ meropenem for 1 month (secondary selection). Transformation

efficiency was recorded after 1 month of secondary selection as the percentage of initial protocorms yielding survived PLBs from 3 replications experiments. Data collected were subjected to statistical analysis after arcsine transformation and were compared using one way ANOVA, followed by tukey's multiple range test using SPSS program (IBM SPSS statistical version 22).

After secondary selection, PLBs were transferred onto ND medium containing 10 mg L⁻¹ hygromycin and 10 mg L⁻¹ meropenem for shoot formation and rooting. Some putative transgenic plantlets were transferred to pots and grown in growth chamber at 25°C under a 16-/8-h (day/night) photoperiod without any acclimatization treatment.

GUS assay

After 5 months of selection, hygromycin-resistant plantlets were randomly selected and assayed for GUS gene Histochemical GUS assay (Jefferson, 1987) with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) as the substrate. Leaves and roots of control and transformed plantlets were incubated in sodium phosphate buffer containing X-Gluc overnight at 37°C after vacuum-infiltration with the buffer solution using desiccator for 15 min. Following the incubation, tissues were bleached with 70% ethanol until chlorophyll was removed.

DNA isolation, polymerase chain reaction (PCR) and Southern hybridization

Total DNA was extracted from leaves (1.5 g fresh weight) of the control and putative transgenic plants following CTAB method (Murray and Thompson 1980). PCR detection of *hpt* and *gus* was performed as described by Hamill et al. (1991) and Xiao and Ha (1997), respectively. The PCR amplifications were carried out using the following thermal cycles: 30 cycles of 94°C for 1 min (denaturation), 59°C for *hpt* and 62°C for *gus* for 1 min (annealing) and 72°C for 1.5 min (elongation). Primers used for amplifying a 0.6-kb fragment inside the *hpt* gene were 5'-ACAGCGTCTCCGACCTGATGCA-3' and 5'AGTCAATGACCGCTGTTATGCG-3' and those amplifying a 1.2 kb fragment of the *gus*

gene were 5'-GGTGGGAAAGCGCGTTACAAG-3' and 5'-GTTTACGCGTTGCTTCCGCCA-3'. After amplification, 3 µl of PCR products were loaded on the gel and detected by ethidium bromide staining after electrophoresis on 1% agarose gel at 100 V for 35 min. For Southern hybridization, 10 µg of genomic DNA was digested overnight with *Hind*III, electrophoresed on 0.9% agarose gel, and subsequently transferred to a nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co, Billerica, MA, USA). The GUS probe (1.2 kb) was generated from plasmid DNA of pIG121Hm by labeling with digoxigenin (DIG) using the PCR DIG Probe synthesis kit (Roche Diagnostics, Mannheim, Germany) and the following set of primers, 5'-GGTGGGAAAGCGCGTTACAAG-3' and 5'-GTTTACGCGTTGCTTCCGCCA-3' were used. Washing and detection were carried out according to the instruction manual of the DIG labeling and detection system (Roche Diagnostics, Mannheim, Germany). For detection of hybridization signals, the membrane was exposed to a detection film (Lumi-film Chemiluminescent Detection Film; Roche Diagnostics, Mannheim, Germany) for 60 min.

Results and discussion

Transformation and selection of hygromycin-resistant protocorms

Our preliminary study showed that prolonged co-cultivation period of 4 days caused rapid browning of protocorms in *D. nobile*-type hybrid after transfer onto selection medium, whereas no appreciable damage was observed in the co-cultivation of up to 3 days. After 3 days of co-cultivation, used in the present study, bacterial overgrowth was observed around protocorms infected for 300 min at 1:10 bacterial concentration (Figure 2-1A), whereas protocorms infected for 30 min did not show bacteria overgrowth irrespective of the bacterial concentration. In both cases, some of the infected protocorms continued to grow and retained their green color after transfer onto selection medium containing antibiotics, while all of the control protocorms without *Agrobacterium* infection failed to grow and turned brown within

4 weeks of culture on selection medium. Although bacterial overgrowth during the selection stages is usually considered as a serious problem in *Agrobacterium*-mediated genetic transformation (Arifin et al. 2004), it was successfully prevented by using 20 mg L⁻¹ meropenem in the present study even if the overgrowth was observed during the co-cultivation period. Previously, Mishiba et al. (2005) succeeded to obtain transgenic plants in *Phalaenopsis* by infecting 21 day-old protocorms with the same *Agrobacterium* strain, EHA101 (pIG121Hm). Although we did not examine the optimum stage of protocorm development for *Agrobacterium* infection, present results clearly indicate the appropriateness of the selected developmental stage of protocorms as the target material in *D. nobile*-type hybrid.

In the present study, inoculation time of *Agrobacterium* solution gave apparently high effect on transformation efficiency and long inoculation time of 300 min resulted in higher transformation efficiencies (23-27%) than short inoculation time of 30 min, which gave 13-14% efficiencies (Table 2-1). On the contrary, Men et al. (2003b) obtained the highest transformation efficiency by infecting PLBs of *D. nobile* with AGL1/pCAMBIA1301 vector with the short inoculation time (30 min) and indicated that the long period of 60 min significantly decreased transformation to 3-4%. In *Phalaenopsis*, bacterial inoculation period of 7 h was used for production of transgenic plants when protocorms were used as a target (Mishiba et al. 2005), whereas 2 h was found to be optimal for the transformation of PLBs (Sjahrill and Mii 2006). These results suggest that the long period of inoculation is suitable for transformation of orchids when using protocorms as target materials. Although the reason for the difference in the optimum time of infection with *Agrobacterium* for transformation efficiency between protocorm and PLB, it might be attributed to the differences in the strain of *Agrobacterium* or in the susceptibility between these two types of target materials.

Although usefulness of high bacterium concentration has been reported as an imported factor for achieving transformation of orchids (Mishiba et al. 2005; Chin et al. 2007; Shrestha et al.

2007; Zhang et al. 2010), it has less effect in the present study, i.e. 1:10 dilution ($OD_{600} \approx 0.1$) gave slightly higher transformation efficiency than 1:50 dilution only at 300 min inoculation time and no differences was found at 30 min inoculation (Table 2-1).

Confirmation of transformation

The surviving protocorms obtained from the primary selection were cut into 2 pieces and cultured on recovery medium lacking hygromycin. One month after the culture on this medium, PLBs were regenerated from the surviving tissues of protocorms. Some of PLBs survived after transfer onto the same selection medium used for the secondary selection of the transgenic tissues and regenerated into shoots (Figure 2-1B). They produced roots after transfer to medium containing reduced concentration of hygromycin to 10 mg L^{-1} and the same concentration (10 mg L^{-1}) of meropenem (Figure 2-1C).

The leaf and root tissues of plantlets regenerated on the secondary selection medium showed positive results for histochemical GUS assay (Figure 2-2). About 90% of the selected plants showed GUS staining in these tissues (data not shown), whereas GUS activity was not detected in any of the tissues of untransformed plantlets. To confirm the presence of transgenes in the putative transformants, PCR analysis was carried out for the *hpt* and *gus* genes. All selected hygromycin-resistant plants showed positive amplification for both genes (Figure 2-3A), which were not detected in the untransformed plants, indicating that the T-DNA of the binary vector was successfully integrated into the genome of the transgenic plants. Southern hybridization was also performed on randomly selected plantlets using *GUS* probe. All the plants showed hybridization signals with the integration of one to five copies of T-DNA in the plant genome (Figure 2-3B, lanes 1-10), while no hybridization signal was observed in the untransformed plant (Figure 2-3B, Lane C).

In conclusion, we have established a highly efficient transformation system with 27.3% transformation efficiency in *D. nobile*-type hybrid using protocorms as target material by applying 300 min period of inoculation of *Agrobacterium* solution at $OD_{600} \approx 0.1$. The value is

much higher than the highest transformation efficiencies of *Dendrobium* obtained previously by 18% with *Agrobacterium*-mediated (Men et al. 2003b) and 19.87% with microprojectile bombardment methods, respectively (Suwanaketchanatit et al. 2007). Therefore, the transformation procedure established in the present study could be used efficiently to introduce various desirable traits such as novel flower colors and disease resistances into *D. nobile*-type hybrids with various genetic background at the same time. Although the reason for the high transformation efficiency of protocorms in *D. nobile*-type hybrid is still unclear, protocorms might also be useful material to obtain comparably high transformation efficiency in other orchid species of *Dendrobium* and other economically important orchid genera. For producing *D. nobile*-type hybrids with blue flower introduction of flavonoid 3', 5'-hydroxylase gene is now in progress.

Acknowledgement

We thank Mr. Nobuyuki Asai, Asai Daikeikan Co. Ltd. to kindly provide the seeds as plant materials in this research.

Table 2-1. Effect of bacterial concentration and inoculation time on transformation efficiency of protocorms in *Dendrobium nobile*.

Inoculation time (min)	Bacterial concentration	Total No. of protocorm	Total No. of Hm-resistant protocorms	Regenerated Hm-resistant protocorms (%)
30	1/10	510	71	13.8±0.5 ^c
	1/50	379	52	13.4±0.5 ^c
300	1/10	515	139	27.3±0.5 ^a
	1/50	453	103	22.8±1.2 ^b

Transformation efficiency was recorded after secondary selection on medium containing 30 mg L⁻¹ hygromycin and 10 mg L⁻¹ meropenem as the number of independent Hm resistant protocorms with respect to the initial number of protocorms infected.

Means of 3 replications with the different letters show significant difference as analyzed by Tukey's test at $p < 0.05$.

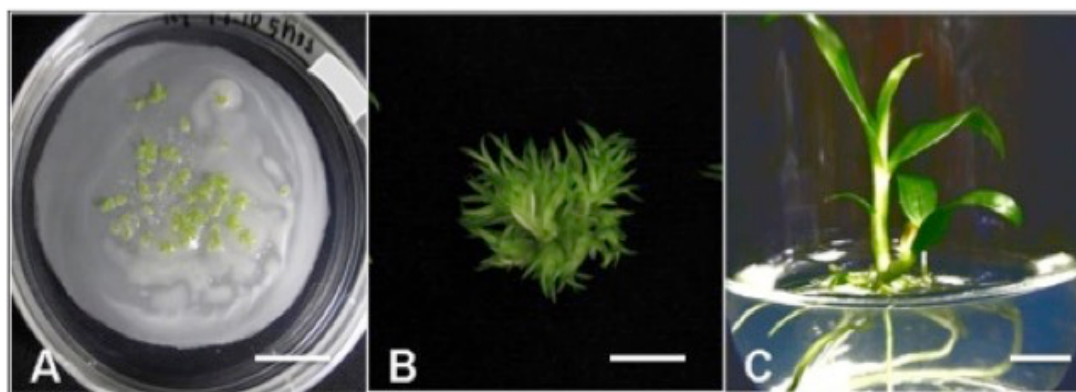


Figure 2-1. Regeneration of putative transgenic plantlets of *Dendrobium nobile*-type hybrid. A: Inoculated protocorms with *A. tumefaciens* (EHA101/pIG121Hm) showing bacterial overgrowth after 3 days of co-cultivation. B: Multiple shoots regenerated from protocorm-derived PLBs after secondary selection with 30 mg L⁻¹ hygromycin and 10 mg L⁻¹ meropenem for 4 months. C: Putative transgenic plantlet 8 months after transfer one of the multiple shoots and culture on medium containing 10 mg L⁻¹ hygromycin and 10 mg L⁻¹ meropenem. Bars = 1 cm.

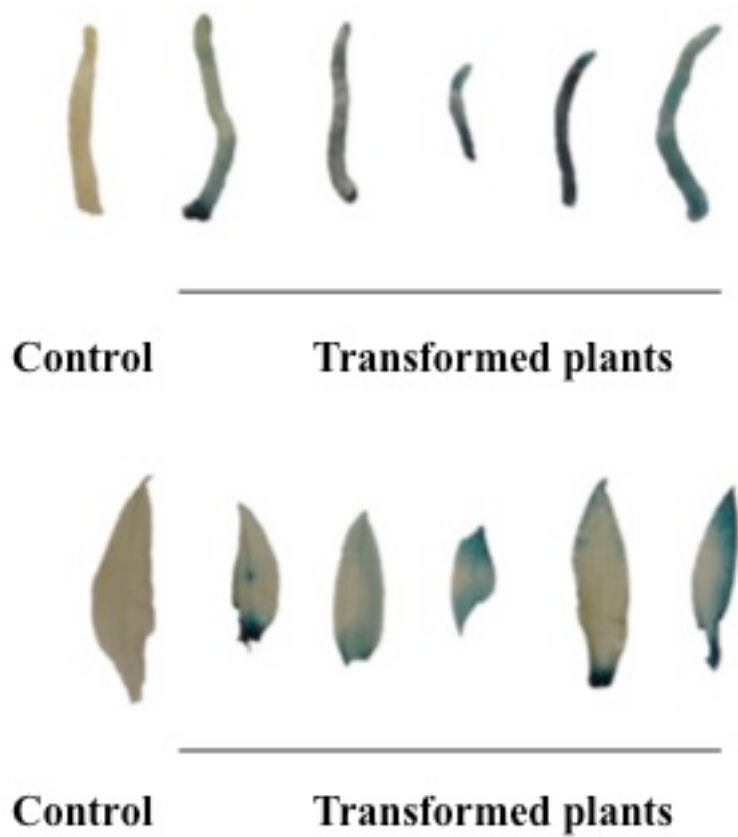


Figure 2-2. GUS histochemical assay of roots (upper) and leaves (lower) of transgenic *Dendrobium nobile*-type hybrid.

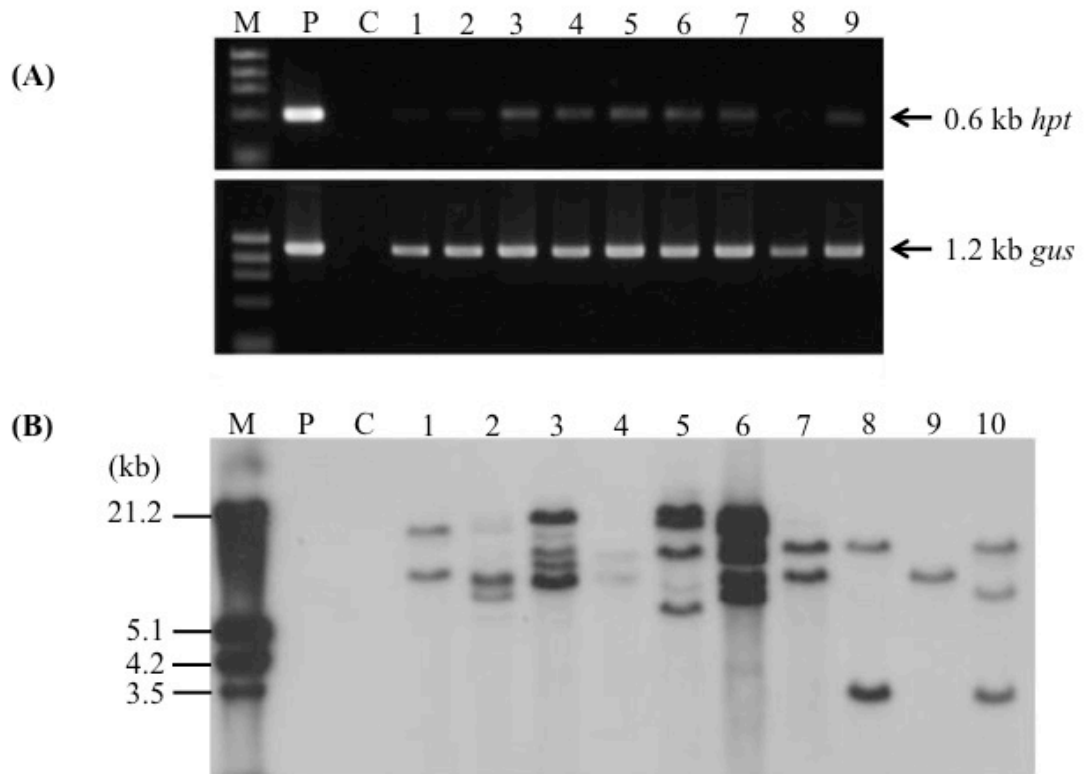


Figure 2-3. Molecular analysis of transgenic *Dendrobium nobile*-type hybrid. (A) PCR analysis of transgenic plantlets for hygromycin phosphotransferase (*hpt*) and β -glucuronidase (*gus*) genes. Lane M: Molecular size marker (ϕ X174/*Hae*III), Lane P: Plasmid pIG121Hm (Positive control), Lane C: untransformed plant as negative control (non-transformed plant), Lanes 1-9: Transgenic plants. (B) Southern blot analysis of transgenic plants. Ten μ g of genomic DNA was digested with *Hind*III and hybridized with digoxigenin (DIG)-label *gus* gene probe. Lane M: Molecular size marker (DIG), Lane C: untransformed plant as negative control (non-transformed plant), Lanes 1-10: Transgenic plants.

Chapter 3

***Agrobacterium*-mediated transformation of protocorm-like bodies
in *Dendrobium* Formidible ‘Ugusu’**

Chapter 3: *Agrobacterium*-mediated transformation of protocorm-like bodies in *Dendrobium* Formidible ‘Ugusu’

Introduction

Dendrobiums are commercially important orchids cultivated as potted plants and cut flowers around the world because of their beautiful flowers and good shape. Nowadays, numerous cultivars have been developed through intra- and inter-specific cross hybridizations. However, improvements are still required in various traits such as flower longevity, disease resistance and novel flower color in order to catch up with its increasing demand and extended market. For achieving these breeding goals, conventional breeding technologies are rather difficult to apply, and genetic transformation remains an useful and possible option.

In *Dendrobium* orchids, genetic transformation studies has been initiated by using biolistic-mediated method (Kuehnle and Sugii 1992; Chia et al. 1994; Tee et al. 2003; Suwanaketchanatit et al. 2007), followed by *Agrobacterium*-mediated method (Men et al. 2003). Among these studies, successful regeneration of transgenic plants has been reported by Men et al. (2003) and Suwanaketchanatit et al. (2007). Based on these studies, virus resistant plants were successfully produced by introducing viral coat proteins in the genome of *Dendrobium* (Chang et al. 2005). The inhibition of ethylene production in order to delay the senescence and increase the flower longevity in *Dendrobium* has also been reported recently (Zheng et al. 2012).

Dendrobium Formidible is a name given to the interspecific hybrid between *D. formosum* and *D. infundibulum* in 1967 (The International Orchid Register, Royal Horticultural Society, <http://apps.rhs.org.uk/horticulturaldatabase/orchidregister/>). Most cultivars of Formidible

have large white flowers (ca. 12 cm in diameter) with yellow eye and usually bloom during early to mid summer season. Moreover, each flower lasts long for more than one month. Due to these unique characters, it has become an important popular pot plant during the hot summer season. Since *D. Formidable* lacks color variations and novel cultivars with other flower colors are now expected because of difficulty in crossing with other *Dendrobium* species and cultivars, genetic transformation technology is now expected to apply to achieve such breeding goals. In the present study, we developed a simple and efficient system for *Agrobacterium*-mediated transformation in *D. Formidable* 'Ugusu', which is a dwarf cultivar suitable as pot plant, by using protocorm-like bodies (PLBs) as a target material.

Materials and Methods

Plant materials

PLBs of *Dendrobium* Formidable 'Ugusu' were induced by culturing shoot tips (1 mm high x 2 mm diameter) excised from sprouting shoots of ca. 10 cm on New Dogashima (ND) medium (Tokuhara and Mii 1993) containing 10 g L⁻¹ maltose, 0.1 mg L⁻¹ 1-naphthaleneacetic acid (NAA), 1.0 mg L⁻¹ N⁶-benzyladenine (BA) and 0.25% gellan gum at pH 5.4 and were subculture every 4 weeks for 2 years. For each subculture, PLBs were cut into several pieces of approximately 3-4 mm in size and transferred to the same fresh medium but added with 10 g L⁻¹ potato granules (Basic American Foods, California, U.S.A). One month-old PLBs after each subculture were used for the transformation experiments. To investigate the effect of the age of PLBs after subculture on transformation, PLBs maintained for the different periods (0, 1, 2, 3, 4, 5 and 6 weeks) after subculture on the same medium were used for the experiment. All of the cultures were incubated at 25°C under constant illumination with cool-white-fluorescent lamps (Hitachi FLR40SW/M/36-B 25HA, Japan).

Plasmid vector and bacterial strain

Agrobacterium tumefaciens strain EHA 101 (Hood et al. 1986) harboring the binary vector plasmid pIG121Hm (Ohta et al. 1990) was used for the transformation. The T-DNA region of pIG121Hm contains hygromycin phosphotransferase gene (*hpt*) and neomycin phosphotransferase II gene (*nptII*) as selectable markers and an intron-containing β -glucuronidase (*gus*) reporter gene.

Inoculation and cocultivation with *Agrobacterium*

Agrobacterium was grown overnight at 28°C in LB liquid medium containing 50 mg L⁻¹ hygromycin (Wako Pure Chemical Industries, Osaka, Japan), 50 mg L⁻¹ kanamycin (Wako Pure Chemical Industries, Osaka, Japan) and 25 mg L⁻¹ chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA). The bacterial suspension was diluted to final density of OD₆₀₀≈1.0 in inoculation medium, which was liquid ND medium containing 100 μ M acetosyringone (AS) (3', 5'-dimethoxy-4'-hydroxy-acetophenone; Sigma-Aldrich, St. Louis, MO, USA). For inoculation, PLBs of 3-4 mm in size were transferred to 35 ml inoculation medium in a 100 ml flask, to which *Agrobacterium* suspension was added to give 1/10 or 1/50 concentration of bacteria (OD₆₀₀=0.1 or 0.02), and incubated for either 30, 45 or 60 min with or without rotation for shaking at 100 rpm. Effect of the shorter inoculation times, i.e., 10, 20, 30 and 40 min, on the transformation efficiency was also examined under optimal concentration of bacteria (OD₆₀₀=0.1) with the rotary shaking. For selection of the suitable inoculation medium, PLBs were infected with *Agrobacterium*, which was suspended in either distilled water (DW), 1% maltose solution without ND nutrient (only 1% Mal) or full strength ND medium with 1% maltose. AS was added to these 3 kind of inoculation media at 100 μ M. The inoculated PLBs were then blotted dry, and co-cultivated on 2.5g L⁻¹ gellan gum (Wako Pure Chemical Industries, Osaka, Japan)-solidified ND medium supplemented with 100 μ M AS at 25°C in the dark for 3 days. After determining the optimum inoculation condition for the transformation, the suitable stage of PLBs for the transformation was investigated.

***Agrobacterium* elimination, selection and plant regeneration**

After cocultivation, the inoculated PLBs were washed with liquid ND medium, blotted dry and transferred onto 2.5 g L⁻¹ gellan gum-solidified ND medium containing 20 mg L⁻¹ hygromycin and 20 mg L⁻¹ meropenem (Ogawa and Mii 2004, 2007; Meropen; Dainippon Sumitomo Pharma, Osaka, JPN) for selection and bacterium elimination (primary selection). PLBs were subcultured every 2 weeks to a fresh medium of the same composition. Secondary PLBs showing green coloration were separated from the inoculated original PLBs by forceps after 2 months of culture on the selection medium and cultured on hygromycin-free fresh medium for 1 month. Thereafter, the PLBs were returned onto the selection medium as used for the primary selection for 2 months. For plantlet regeneration, surviving PLBs were transferred onto gellan gum-solidified ND medium containing 10 mg L⁻¹ hygromycin and 10 mg L⁻¹ meropenem. Regenerated plantlets as putative transformants were each transferred into a culture bottle containing the same selection medium. Three months after transplanting, plantlets which developed 3-4 leaves and several roots were acclimatized in sphagnum moss of small pots and grown in a growth chamber at 25°C under a 16-/8-h (day/night) photoperiod. Transformation efficiency was recorded after 4 month of culture on the secondary selection medium as the percentage of initial PLBs yielding survived PLBs. The experiment was replicated 3 times and the data were subjected to statistical analysis after arcsine transformation and compared using one way ANOVA, followed by Tukey's multiple range test using SPSS program (IBM SPSS statistical version 22).

GUS assay

GUS histochemical assay was performed on hygromycin-resistant PLBs, shoots, leaves, roots and untransformed tissues as a control. These tissues were immersed in X-Gluc (5-bromo-4-chloro-3-indolyl-D-glucuronide) solution (Jefferson, 1987), vacuum-infiltrated for

15 min and incubated overnight at 37°C. The tissues were then soaked in 70% ethanol for several hours to remove chlorophyll before observation.

DNA isolation and molecular analysis

Plantlets regenerated from PLBs were selected randomly to check for the presence of transgene. Total genomic DNA was extracted from leaf tissues (2.0 g fresh weight) of the plantlets using the CTAB method (Murray and Thompson 1980). For detecting *hpt*, *nptII* and *gus*, the PCR amplifications were carried out using the following thermal cycles: 30 cycles with 94°C for 1 min (denaturation), 59°C for *hpt*, 58°C for *nptII* and 62°C for *gus* for 1 min (annealing), respectively and 72°C for 1.5 min (elongation). Primers used for amplifying a 0.6-kb fragment inside the *hpt* gene were 5'-ACAGCGTCTCCGACCTGATGCA-3' and 5'-AGTCAATGACCGCTGTTATGCG-3'; those amplifying a 0.7-kb fragment inside the *nptII* gene were 5'-GAGGCTATTCGGCTATGACTG-3' and 5'-ATCGGGAGCGGCGATACCGTA-3', and those for amplifying a 1.2-kb fragment of the *gus* gene were 5'-GGTGGGAAAGCGCGTTACAAG-3' and 5'-GTTTACGCGTTGCTTCCGCCA-3', respectively. After amplification, 3 µl of PCR products were loaded on the gel and detected by ethidium bromide staining after electrophoresis on 1% agarose gel at 100 V for 35 min.

For Southern hybridization, 10 µg of genomic DNA was digested overnight with *HindIII*, electrophoresed on 0.9% agarose gel, and transferred to a nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co, Billerica, MA, USA). The *hpt* probe (0.6 kb) was generated from plasmid DNA of pIG121Hm by labeling with digoxigenin (DIG) using the PCR DIG Probe Synthesis kit (Roche Diagnostics, Mannheim, Germany). Southern blot hybridization and detection were performed using digoxigenin-labeled *hpt* probe following the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Results and Discussion

Effect of bacterial concentration, inoculation time and inoculation condition on the transformation efficiency

PLBs were infected with two concentrations of *A. tumefaciens* ($OD_{600} = 0.1$ or 0.02) at different inoculation time (30, 45, 60 min) with or without rotary shaking during inoculation. As the results, higher concentration of bacteria, shorter inoculation period and application of agitation gave beneficial effects on the transformation and the highest transformation efficiency of 12.2% was obtained when 1:10 ($OD_{600} = 0.1$) bacterial concentration and 30 min inoculation time was applied with shaking during inoculation (Table 3-1). Especially at this bacterial concentration and inoculation period, 1.8 times higher transformation efficiency was obtained with the application of agitation during the inoculation period than that without it. These results indicate that agitation treatment increased the susceptibility of explants to *Agrobacterium* through giving the damage of the surface of PLBs. For confirming the optimum period of inoculation, PLBs were further inoculated with *Agrobacterium* at the periods for 10 to 40 min at the bacterial concentration of $OD_{600} = 0.1$ with agitation. The results showed that 30 min of inoculation time gave the highest transformation efficiency of 13.9%, whereas shorter inoculation time as 10 and 20 min decreased to 2.8 and 5.6%, respectively and also longer inoculation time as 40 min decreased the efficiency to 6.9 % (Figure 3-1). These results indicate that 30 min was the optimum period for the infection of PLBs with *Agrobacterium* at $OD_{600} = 0.1$ with agitation treatment during inoculation period. In other *Dendrobium* orchids, inoculation of PLBs for 30 min also gave the highest transformation efficiency (Yu et al. 2001; Men et al. 2003). However, in other orchids, combinations of higher bacterial concentration and longer inoculation period have been recommended for the successful genetic transformation, such as $OD_{600} \approx 0.5$ for 4 h in *Vanda* (Shrestha et al. 2007) and $OD_{600} \approx 0.6$ for 4h in *Cattleya* (Zhang et al. 2010), respectively.

Therefore, *Dendrobium* orchids might be more susceptible to *Agrobacterium* infection compared to other kind of orchids.

Effect of different stage of PLBs after subculture and inoculation medium on transformation efficiency

In genetic transformation of various orchids, PLB has predominantly been used as target material for inoculation of *Agrobacterium* because it has high potential to regenerate into plants and could be easily obtained by culturing shoot tip (Tokuhara and Mii 1993; Martin et al. 2005; Shrestha et al. 2007) and root tip (Guo et al. 2010). In the present study, we investigated the suitable stage of PLBs for transformation. Among the 7 stages examined, PLBs obtained 3 weeks after subculture produced the highest transformation efficiency of 16.3% (Figure 3-2). It has been reported that transgene integration is favored in cells at the S- and G1-phase and target tissue in transformation must be actively dividing cells, which could be obtained by sub-culturing (Suwanaketchanatit et al. 2007). Julkifle et al. (2012) have reported that the cells in PLBs are actively dividing at the third week after subculture. Therefore, 3 week-old PLBs might be suitable as a target material for *Agrobacterium* infection.

The composition of inoculation medium has been known to influence the efficiency of transformation. In *Lilium*, Azadi et al. (2010) attempted to investigate the efficiency of transformation by removing some elements in Murashige and Skoog medium (MS) used for inoculation and co-cultivation. The highest transformation efficiency (25.4%) was obtained when calli were inoculated with *Agrobacterium* in MS medium lacking KH_2PO_4 , NH_4NO_3 , KNO_3 and CaCl_2 . However, the inoculation medium with only distilled water (D.W.) and sucrose also showed high transformation efficiency of 10.7%. In the present study, medium containing only 10 g L^{-1} maltose gave slightly but not significantly higher transformation efficiency of 18.5% than that with normal ND medium (17.0%) (Figure 3-3), indicating that

removal of ND mineral and organic components did not affect the transformation efficiency. In contrast, complete removal of medium components from the inoculation medium, i.e., only D. W., gave significantly lower transformation efficiency (6.7%), suggesting the importance of maltose during infection period for the successful transformation with *Agrobacterium*. Previous studies demonstrated that sugars greatly increased the expression of *vir* genes in combination with AS (Shimoda et al. 1990, Wise et al. 2005). In the present study, difference in the presence of maltose in medium was made only at short period of inoculation but not at subsequent cocultivation period. Therefore, effectiveness of maltose for *Agrobacterium* infection was exerted probably through its enhanced attachment to the host cells. For achieving more efficient transformation, it might be necessary to find out more suitable sugars for *Dendrobium* as shown in lily transformation (Azadi et al. 2010).

Selection and regeneration of transgenic plants

When PLBs 3 weeks after subculture (Figure 3-4A) were cultivated for 2 months on the selection medium containing 20 mg L⁻¹ hygromycin after co-cultivation (primary selection), some parts of them remained green (Figure 3-4B), whereas others turned brown and died. The survived PLB tissues were separated and cultured for one month on hygromycin-free ND medium on which they initiated to induce secondary PLBs. These PLBs with new proliferation were reselected by transferring and subculturing on the primary selection medium i.e. ND medium containing 20 mg L⁻¹ hygromycin at one month intervals. One month after the transfer, all of the secondary PLBs survived (Figure 3-4C) and produced multiple shoots after 4 months of culture on medium (Figure 3-4D). When these multiple shoots were transferred onto medium containing reduced concentration of hygromycin to 10 mg L⁻¹, they produced roots and developed into plantlets with 3-4 leaves 6 months after the transfer (Figure 3-4E). These plantlets were successfully acclimatized in sphagnum moss of

small pots in a growth chamber at 25°C under a 16-/8-h (day/night) photoperiod (Figure 3-4F).

GUS histochemical assay and confirmation of transgenic plants

Hygromycin resistant PLBs and multiple shoots showed histochemical GUS staining, which was not observed in untransformed control PLBs (Figure 3-5). GUS staining could also be detected in shoots, leaves and roots of the regenerated plantlets. The presence of the *gus*, *nptII* and *hpt* genes in these putatively transformed plantlets was confirmed by PCR amplification, which showed the expected 1.2-, 0.7- and 0.6-kb amplification products, respectively (Figure 3-6A). Furthermore, Southern blot analysis using the *hpt* gene probe showed that all of the plantlets confirmed the presence of the *hpt* gene sequence in the genomes of all the 6 plantlets randomly selected. Of these six transgenic plantlets, 3 exhibited single insertion sites (Lanes 2, 3 and 5), whereas another 3 showed 2 (Lane 4) or more (Lanes 1 and 6) T-DNA insertion sites in their genomes (Lane 1) (Figure 3-6B).

In conclusion, we have succeeded in establishing an efficient transformation system for *D. Formidible* 'Ugusu' via *Agrobacterium*-mediated transformation. The factors optimized here, use of 3 week-old PLBs after regular subculture, appropriate inoculation medium (1% maltose+100 µM AS), and optimum bacterial concentration ($OD_{600} = 0.1$) and inoculation time (30 min) could be applied for the transformation of various cultivars of *Dendrobium* and their hybrids. Introduction of the genes that regulate economically important traits such as flower color and disease resistance into *D. Formidible* 'Ugusu' are now in progress.

Table 3-1. Effect of bacterial concentration and inoculation time on transformation of *Dendrobium Formidible* 'Ugusu'.

Inoculation condition	Conditions for transformation system		Total No. of PLBs inoculated	No. of Hm-resistant PLBs ¹⁾	Transformation efficiency ²⁾
	Bacterial concentration	Inoculation time (min)			
Shaking	1:10	30	90	13	12.2 ± 0.5 ^a
		45	90	7	6.7 ± 0.5 ^b
		60	90	6	4.4 ± 1.1 ^{bc}
	1:50	30	90	6	5.5 ± 1.0 ^{bc}
		45	90	6	3.4 ± 0.2 ^c
		60	90	4	3.3 ± 0.1 ^c
Non-shaking	1:10	30	90	4	6.7 ± 0.4 ^b
		45	90	4	5.6 ± 1.1 ^{bc}
		60	90	4	4.4 ± 1.0 ^{bc}
	1:50	30	90	3	3.4 ± 0.2 ^c
		45	90	5	4.4 ± 0.9 ^{bc}
		60	90	4	4.7 ± 1.5 ^{bc}

Transformation efficiency was recorded as the percentage of PLBs that produced shoot primordia after 4 months of secondary selection on medium containing 20 mg L⁻¹ hygromycin and 20 mg L⁻¹ meropenem.

¹⁾ Recorded 4 months after transfer onto secondary selection medium.

²⁾ Means of 3 replications with the same letters show significant difference as analyzed by Tukey's test at $p < 0.05$ after arcsine transformation of the data.

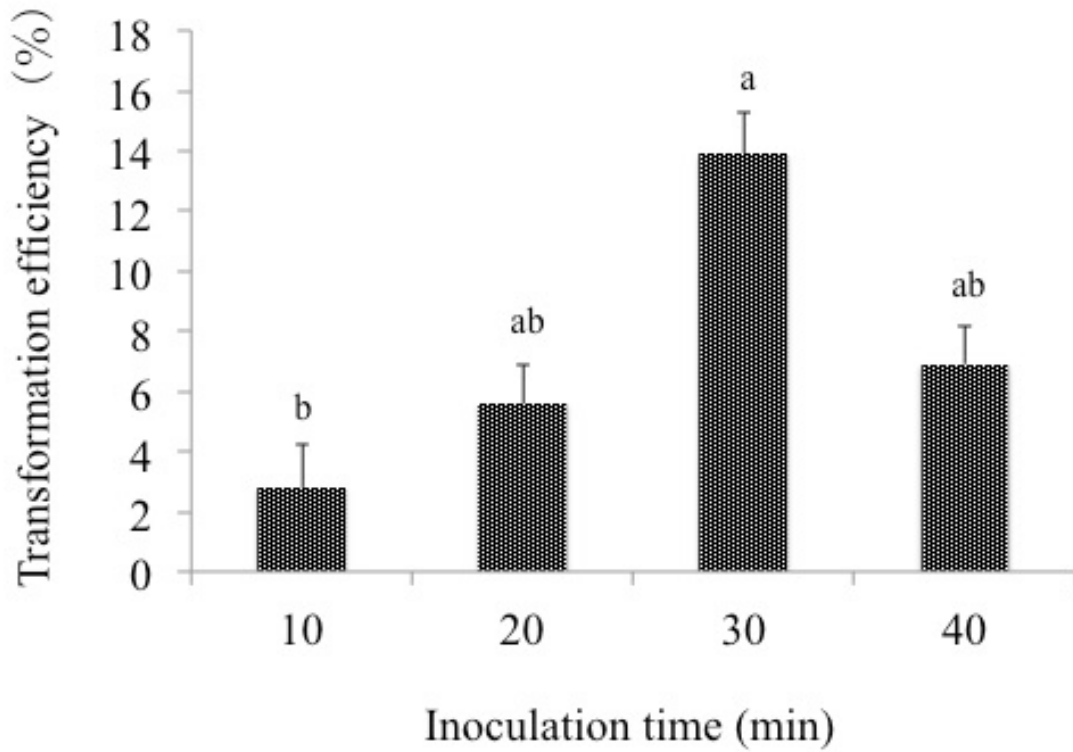


Figure 3-1. Effect of the inoculation time of PLBs with *Agrobacterium* on transformation efficiency of *Dendrobium* Formidible 'Ugusu'.

Means of 3 replications with the different letters show significant differences as analyzed by Tukey's test at $p < 0.05$ after arcsine transformation of the data

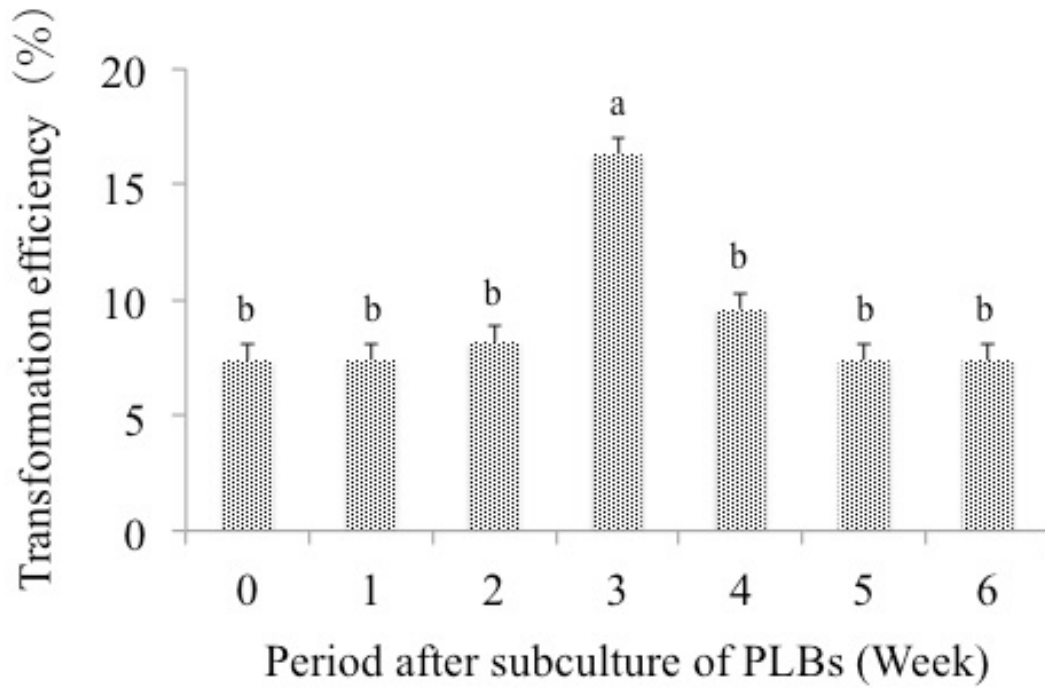


Figure 3-2. Effect of the age of PLBs on transformation efficiency of *Dendrobium* Formidible 'Ugusu'.

Means of 3 replications with the same letters show significant difference as analyzed by Tukey's test at $p < 0.05$ after arcsine transformation of the data.

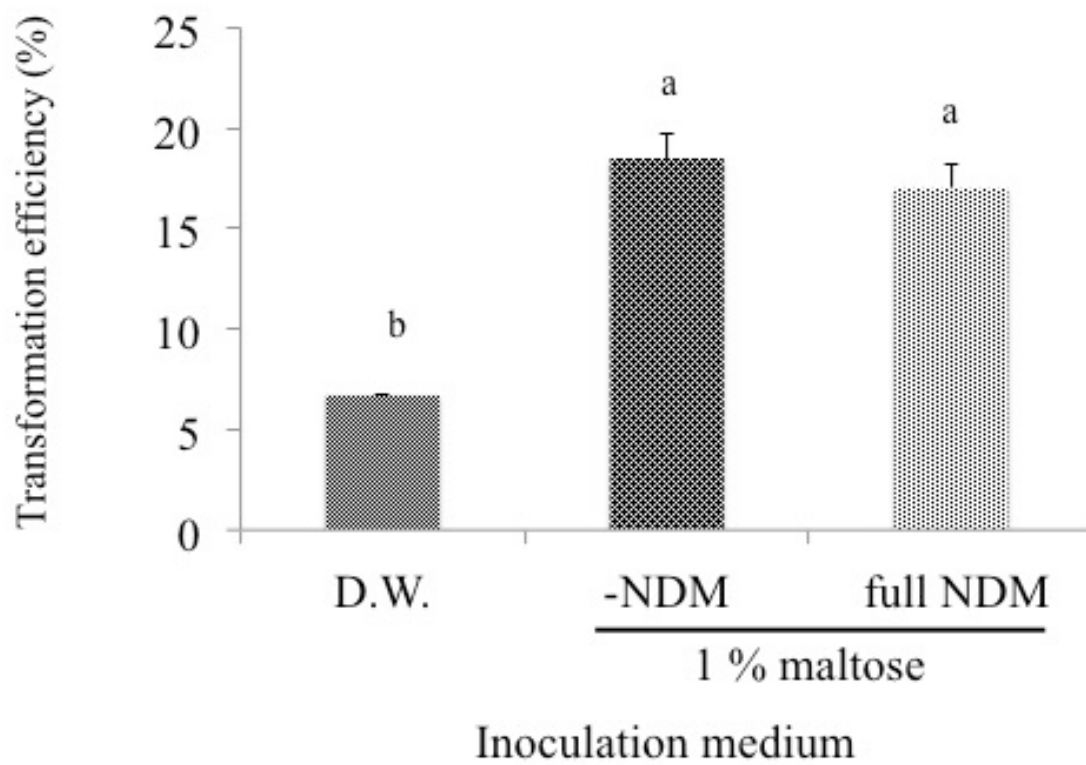


Figure 3-3. Effect of inoculation medium on transformation of *Dendrobium* Formidible 'Ugusu'. For each medium, 100 μ M acetosyringone was added.

Means of 3 replications with the different letters show significant difference as analyzed by Tukey's test at $p < 0.05$ after arcsine transformation of the data.

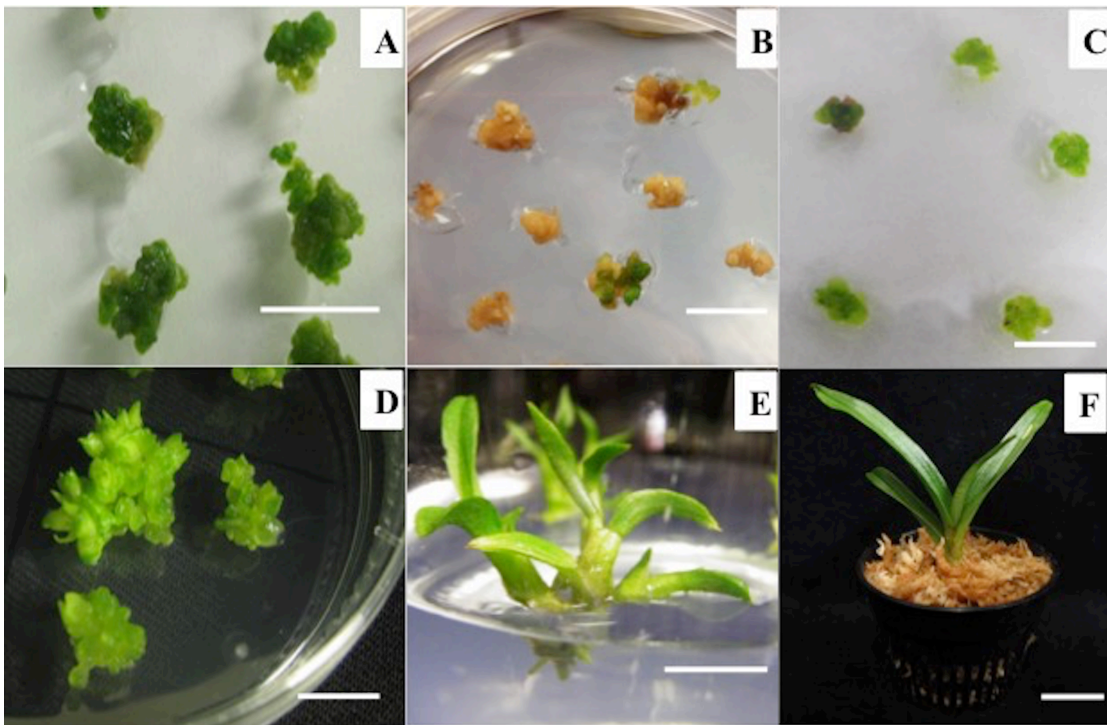


Figure 3-4. Plantlet regeneration of *Dendrobium* Formidible ‘Ugusu’ after infection of PLBs with *A.tumefaciens* (EHA101/pIG121Hm) and selection with hygromycin.

A: PLBs after subculture for 3 weeks on ND medium containing 0.1 mg L^{-1} 1-naphthaleneacetic acid (NAA), 1.0 mg L^{-1} N6-benzyladenine (BA), 1% maltose and 0.25% gellan gum at pH 5.4. B: Inoculated PLBs with *A.tumefaciens* (EHA101/pIG121Hm) after 6 weeks of culture on selection medium containing 20 mg L^{-1} hygromycin and 20 mg L^{-1} meropenem. C: Surviving PLBs after transfer to secondary selection medium containing 20 mg L^{-1} hygromycin and 20 mg L^{-1} meropenem for 1 month. D: Multiple shoots regenerated from secondary PLBs after secondary selection with 20 mg L^{-1} hygromycin for 4 months. E: Rooting plantlets regenerated from multiple shoots on selection medium with 20 mg L^{-1} hygromycin and 20 mg L^{-1} meropenem for 6 months. F: Successfully acclimatized transgenic plants grown for 3 months in a growth chamber at 25°C under a 16-/8-h photoperiod. Bar = 1 cm

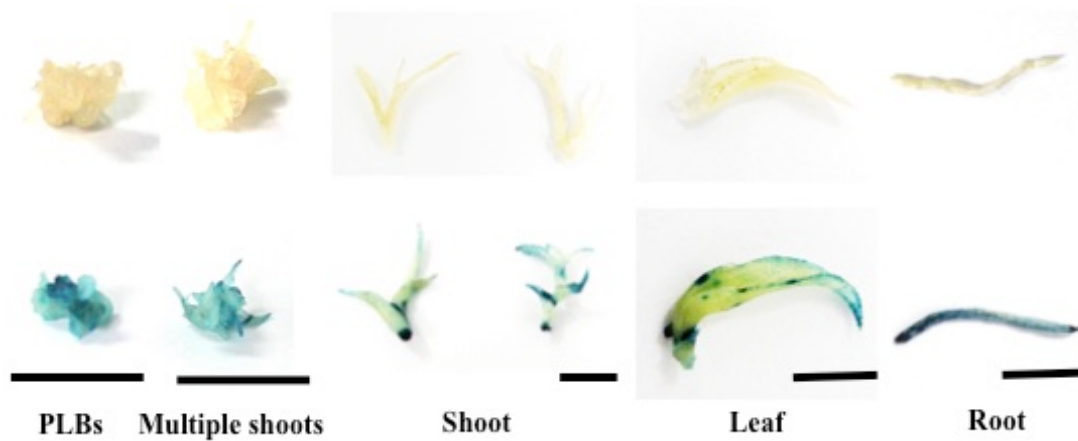


Figure 3-5. GUS histochemical assay of *Dendrobium* Formidible 'Ugusu' at different growth stages

PLBs: after selection on medium containing 20 mg L^{-1} hygromycin and 20 mg L^{-1} meropenem for 2 months. Multiple shoots: shoots regenerating from PLBs after secondary selection on medium 20 mg L^{-1} hygromycin and 20 mg L^{-1} meropenem for 3 months. Shoot: 5 months after regenerated from PLBs. Leaf and Root: 6 months after regeneration from PLBs. Un-transformed plant (upper) and transgenic plant (lower) Bar = 1 cm

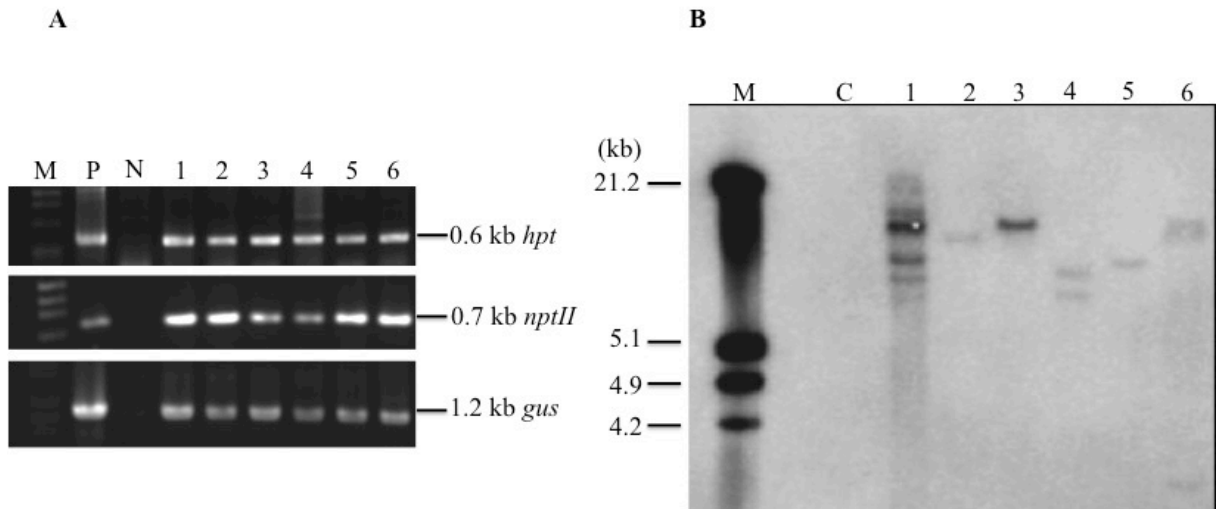


Figure 3-6. Molecular analysis of transgenic *Dendrobium* Formidible ‘Ugusu’.

(A) PCR analysis of transgenic plantlets for hygromycin phosphotransferase (*hpt*), neomycin phosphotransferase II (*nptII*) and β -glucuronidase (*gus*) genes. Lane M: Molecular size marker (ϕ X174/*HaeIII*), Lane PC: Plasmid pIG121Hm (positive control), Lane NC: untransformed plant as negative control (non-transformed plant), Lanes 1-6: Transgenic plants of *D. Formidible* ‘Ugusu’. (B) Southern blot analysis of transgenic plants. Ten μ g of genomic DNA was digested with *HindIII* and hybridized with digoxigenin (DIG)-labelled HPT gene probe. Lane M: Molecular size marker (DIG), Lane C: untransformed plant as negative control (non-transformed plant), Lanes 1-6: Transgenic plants.

Chapter 4

Blue gene expression in *Dendrobium nobile* by introducing flavonoid 3', 5' –hydroxylase gene via *Agrobacterium*-mediated transformation

Chapter 4: Blue gene expression in *Dendrobium nobile* by introducing flavonoid 3', 5' – hydroxylase gene via *Agrobacterium*-mediated transformation

Introduction

Dendrobium nobile is one of the most popular cultivated orchids for house decoration, blooming in winter and spring. This genus is an epiphyte of Asian origin, found throughout tropical and subtropical Asia, as Far East as the Fiji Islands and Southern Australia. It is a widespread species, which occurs in many forms and varieties including waxy and fragrant, with colors ranging from white to pink and purple. Recently, the high demand for *D. nobile* has attracted the attention of biotechnology companies for large scale clonal propagation of elite genotypes of this genus (Faria and Illg 1995). One trait that is prime importance in breeding is the blue color, which lacks germplasm by sexual hybridization. Violet to blue color is produced by the delphinine-based anthocyanin. Flavonoid 3', 5'-hydroxylase (F3'5'H) is one of the key enzymes with potential for delphinidin biosynthesis, however *Dendrobium* lacks the F3'5'H gene. Unfortunately, this gene cannot be manufactured. Genetic engineering is a powerful method that may be able to resolve this problem. Recently, around 20 different ornamental species have been successfully produced with the blue flower and they are now marketed as commercial cultivars in the USA, Europe, Japan, and Australia. *Rosa hybrida* lacks the blue flower variety as it does not contain a delphinidin-based anthocyanin (F3'5'H). In a previous study, delphinidin-accumulating rose was successful in producing by down-regulation, the endogenous dihydroflavonol 4-reductase (DFR) gene. Then, overexpressing of the *Irish* × *Hollandica* DFR gene was done by the introduction of the *viola* F3'5'H gene (Katsumoto et al. 2007).

To introduce the target F3'5'H gene into *Dendrobium*, the F3'5'H needs to be sufficient to engineer the altered flower color. *Commelina communis*, commonly known as the Asiatic Dayflower, is a herbaceous annual plant. Hayashi et al. (1958) reported the blue pigment in *Commelina communis* as commelinin, a metal-complex anthocyanin from the blue petal. However, the existence of a blue-color magnesium complex was denied by Bayer et al. (1996). Moreover, the metal complexation of anthocyanin and intermolecular hydrophobic association explained the blue flower-color development and the stability of the color (Kondo et al. 1992). It is well-known that delphinidin is an important factor for producing blue flower color; the commelinin molecule involves the chelate formation of two Mg²⁺ ions with the 4'-keto-quinoidal base of anthocyanin and coordination with the other two Mg²⁺ ions to produce flavone plays a crucial role in giving stability to the complex molecule (Shiono et al. 2008).

Here, we attempt to introduce the F3'5'H gene isolated from *Commelina cummunis* into *D. nobile*. The expressing of the F3'5'H gene into the different morphology was performed based on the divergent genetic backgrounds as the target material.

Materials and Methods

Plant materials

Seeds of *Dendrobium nobile*, an elite clone obtained from a cross between *D. Flower Palace* 'Cinderella' × *D. Santa Isabel* 'True Love' representative non-anthocyanin source and 'Watarun' × '868' representative as anthocyanin source gene background were kindly provided by Mr. Nobuyuki Asai, Asai Daikeien Co. Ltd (Figure 4-1). To produce protocorms, used system that described in chapter 2. The young protocorm after germinated at 21 days was used as material in this experiment.

Plasmid vector and bacterial strain

Agrobacterium tumefaciens strain EHA 101 (Hood et al. 1986), which harboring with four different binary vectors, which contains a hygromycin phosphotransferase gene (*hpt*) and *Commelina communis* F3'5'H gene both under the control of a 35S cauliflower mosaic virus promoter as a basal gene in vector. Four vectors such as pBIH-35D9-PDB (D 9), pBIH-35D17-PDB (D 17), pBIH-35S-Del17 (Del 17), pBIH-35S-Del18 (Del 18) contain different gene in vectors as described in figure 4-2 were used in this transformation experiment.

Inoculation and co-cultivation with *Agrobacterium*

Four vectors of *Agrobacterium* were grown overnight and cultured in liquid LB medium containing 50 mg L⁻¹ hygromycin at 28°C. The protocorms after germinated for 21 days were used as material in transformation following procedure that described in chapter 2.

Elimination of bacteria, selection and plant regeneration

After co-cultivation, inoculated protocorms were subjected to eliminate and select in the same time. From this step until acclimatize was used procedure as described in chapter 2.

DNA isolation, polymerase chain reaction (PCR) and southern hybridization

Genomic DNA was extracted from leaves of the wild type as a negative control and putative transgenic plants using CTAB method (Murray and Thompson 1980). PCR detection of *hpt* and *F3'5'H* gene was performed to detected the presence gene in the putative transgenic plants. The PCR amplifications were carried out using the following thermal cycles: 30 cycles of 94 °C for 1 min (denaturation), 59 °C for *hpt* or 54 °C for *F3'5'H* for 1 min (annealing) and 72 °C for 1.5 min (elongation). Primers used for amplifying a 0.6-kb fragment inside the *hpt* gene were 5'-ACAGCGTCTCCGACCTGATGCA-3' and 5'-AGTCAATGACCGCTGTTATGCG-3' and those amplifying a 1.5 kb fragment of the *F3'5'H* gene were 5'-ATGGTACCCCTTACGTACCTT-3' and 5'-

TTATGTTGTTTTTATATTCTTATAAACG-3'. After amplification, 3 µl of PCR products was loaded on the gel and detected by ethidium bromide staining after electrophoresis on 1% agarose gel at 100 V for 35 min.

For southern hybridization, 10 µg of genomic DNA was digested overnight with *Hind*III, electrophoresed on 0.9% agarose gel, and subsequently transferred to a nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co, Billerica, MA, USA). The *hpt* probe (0.6 kb) was generated from plasmid DNA of pIG121Hm by labeling with digoxigenin (DIG) using the PCR DIG Probe synthesis kit (Roche Diagnostics, Mannheim, Germany) and the following set of primers, 5'-ACAGCGTCTCCGACCTGATGCA-3' and 5'-AGTCAATGACCGCTGTTATGCG-3'. Washing and detection were carried out according to the instruction manual of the DIG labeling and detection system (Roche Diagnostics, Mannheim, Germany). For the detection of hybridization signals, membrane was exposed to a detection film (Lumi-film Chemiluminescent Detection Film; Roche Diagnostics, Mannheim, Germany) for 60 min.

For a transcription of the transgene, RT-PCR analysis was carried out for the gene expression. Total RNA was extracted from the 1.5 g leaves of the wild type as a negative control and transgenic plants by using extraction buffer method. For the management contaminated DNA, RNA (3µg) was treated with RNase-free DNase (Promega) at 37°C for 30 min before the first-strand synthesis. DNase-treated RNA samples were reverse-transcribed using Superscript III RNase H reverse transcriptase (RT) kit (Invitrogen) with minor modification. Briefly, 2 µg DNase-treated total RNA was mixed with 100 ng random primers, 1 µl 10 mM dNTP mixes and 7 µl diethylidicarbonate (DEPC) treated water. The reaction was performed at 65°C for 5 min and incubated on ice for 1 min. Then, 4 µl of 59 First strand buffer, 1 µl of 0.1 M DTT and 1 µl Superscript III RT was added. Finally, the reaction was incubated for 1 h at 50°C and for 15 min at 70°C. These templates after performed this step were used for amplification of the *F3'5'H* gene with the primer and PCR

cycle as described above in PCR analysis. The control of quality in the sample was used rice actin gene (RAc1; X16280). The PCR amplification was carried out using the primer pair: 5'-GAAAATGGTGAAGGCTGGTTTTG-3' and 5'-AGGATTGATCCTCCGATCCAGA-3'. The PCR products were then separated then divided by 1% agarose gel electrophoresis for 30 min at 100 volts.

Results and discussion

Selection of Hm-resistant PLBs, regeneration and acclimatization of the transgenic plant

After three days of co-cultivation, bacterial overgrowth was observed around two genotype protocorms infected with all vectors similar to *Agrobacterium* infection, as described in Chapter 2. In all vectors between two kinds of materials, some of the infected protocorms continued to grow and retained their green color after transfer onto the selection medium, which contained the antibiotics. All the control protocorms without *Agrobacterium* infection failed to grow, turning brown within four weeks of culture. After remaining on the selection medium, containing 30 mg L⁻¹ hygromycin and 20 mg L⁻¹ meropenem for 6 months, the surviving protocorms that regenerated from the secondary PLBs were recorded for transformation efficiency. In this study a transformation procedure was used that combined the effects of bacterial concentration and inoculation time. Transformation efficiency of 'Cinderella' × 'Truelove' resulted in 4.2, 7.0, 8.6, and 8.8% efficiency for D17, D9, Del17, and Del18, respectively (Table 4-1). Infection of 'Watarun' × '868' showed a slightly lower transformation efficiency of 3.0, 3.1, 3.5, and 3.7 for Del 18, Del 17, D 9, and D 17, respectively. These results were significantly less than the transformation efficiency previously investigated in Chapter 2. However, this experiment showed no significance in the same genotype (Table 4-1), indicating that the transformation efficiency may be affected by the target-introducing gene that introduced the desirable traits. Patterns for selection and regeneration showed no change in the color of the PLBs or shape within selection over 12

months. The purple coloration in the 'Watarun' × '868' PLBs tissue after regeneration in new Dogashima medium (NDM) containing 10 mg L⁻¹ hygromycin and 10 mg L⁻¹ meropenem was observed for 20 months (Figure 4-3). For 'Cinderella' × 'Truelove', the purple coloration in the PLB derived from four protocorms was observed after selection for 24 months. These plants were regenerated from the purple PLB tissue to form shoots and plantlets in the bottle. Both genotypes showed purple stem derived from inoculated protocorms with D 17 (Fig. 4-4).

However, other vectors did not demonstrate the purple color in the shoots. The outer leaves were stripped from the purple shoots, which were then cut lengthways showing the strong purple internal shoot. The leaves also showed the purple color in the plantlets, however another vector was not observed similar to the control (Figure 4-5). To acclimatize the *in vitro* plantlets after vigorous regeneration to over 10 cm in size, they were transferred from gellan gum; the roots were washed and then cultured with sphagnum moss after immersion in D.W. overnight in the greenhouse. The purple plants showed darker greenish and purplish coloration in both the shoots and leaves than the *in vitro* plants in the greenhouse (Fig. 4-6). One line of 'Cinderella' × 'Truelove' (2B2C#10) was a significantly smaller size than the others, like a dwarf, but apart from this line there was no difference in plant size and they could be regenerated as control plants. After a long culture time in the bottle, one line of 'Cinderella' × 'Truelove' (2D2C#5) showed flowers, displaying a bluish coloration, but with a deformed flower (Figure 4-6). This indicated that the F3'5'H gene could be introduced to protocorms, by crossing seeds on the divergent genetic background with lack pigment of anthocyanin (Figure 4-1). Moreover, results suggested that the flower will bloom with a dark purple-like blue, more than the color displayed by the *in vitro* plant, and similar to the acclimatized plants, showing the dark purple on the shoots and leaves (Figure 4-4, 5).

Confirmation of transgene and gene expression in transgenic plants

Putative transformants were subjected to DNA extraction using Cetyl trimethyl ammonium bromide (CTAB) buffer. To confirm the presence of transgenes in the transformants, polymerase chain reaction (PCR) analysis was performed for the gene. All selected hygromycin-resistant plants showed positive amplification of the hygromycin phosphotransferase (*hpt*) gene in all vectors (Fig. 4-7). This was not detected in the untransformed plants, indicating that the T-DNA of the binary vector was present in the genome of the transgenic plants.

Southern hybridization was performed on randomly selected plantlets using *hpt* probe. All plantlets showed hybridization signals with the insertion of 1-3, 1-2, 1-5, and 2-multi copies of T-DNA integrated from D 9, D 17, Del 18, and Del 17, respectively in the plant genome (Figure 4-8, lanes 1-4). No hybridization signal was observed in the untransformed plant (Figure 4-8, Lane C).

To confirm the presence of the target F3'5'H gene, PCR analysis was carried out for the gene. For this confirmation a shoot was used showing purple coloration. All selected hygromycin-resistant plants showed positive amplification of the F3'5'H gene in D 17, Del 17, and Del 18 vectors (Figure 4-9), which was not detected in the D 9 vector and untransformed plants. These results indicated that T-DNA of the binary vector was present in the genome of the transgenic plants, except T-DNA of D 9 vectors.

After confirming the presence of the F3'5'H gene, reverse transcription polymerase chain reaction (RT-PCR) analysis was carried out to perform the transcription of the gene. In the analysis, positive amplification of the F3'5'H gene in Del 17 and Del 18 were detected in the plants (Figure 4-10). The D 17 vectors were detected in only the band of purple shoots (Figure 4-10), and not detected in the normal green shoot, while D 9 vector were still undetected in the band similar to the control. These results indicated the effect of the vectors of the transformation was unstable, while hygromycin-resistance was confirmed. However,

the target gene could not be transcript maybe affected by gene silencing or problems with the vectors.

These studies confirmed the expression of flavonoid 3', 5'-hydroxylase gene, introducing the target gene isolation from *Commelina communis* with *Agrobacterium*-mediated transformation methods to the target material. In addition, we succeeded in producing the blue-purple flower in *D. nobile*. Until the flower blooms in all transgenic, the pattern of the flower, special colors or shape cannot be determined. However, the target gene could be introduced in the target plant that we confirmed in this step. Moreover, transgenic plants could be regenerated by propagation to increase the number of clones with the same phenotype.

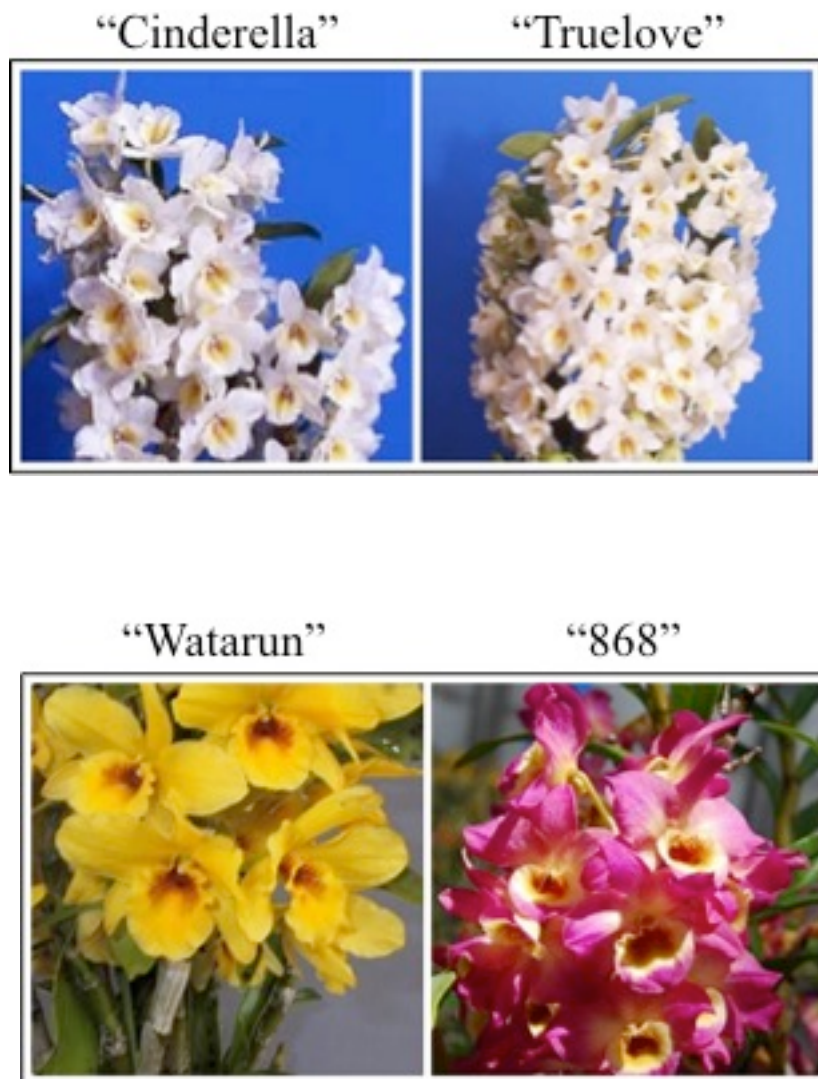


Figure 4-1. Plant material for blue gene transformation

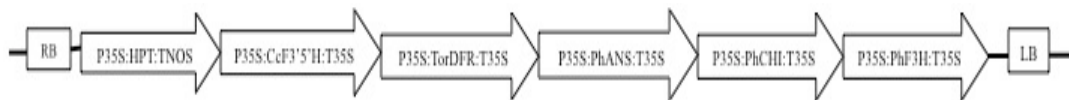
pBIH-35D9-PDB :D9



pBIH-35D17-PDB :D17)



pBIH-35S-Del17 : Del17



pBIH-35S-Del18 : Del18



Figure 4-2. Four different vectors using in introducing F3'5'H gene via *Agrobacterium*-mediated transformation

Table 4-1 Production of hygromycin resistant protocorms after transformation with different vectors.

Seed source	<i>A. tumefaciens</i>	No. of protocorms	No. of Hm-resistant protocorms	Percentage of Hm-resistant protocorms
Cinderella	D 9	370	26	7.0
	D 17	309	13	4.2
	Del 17	163	14	8.6
Truelove	Del 18	341	30	8.8
Watarun	D 9	58	2	3.5
	D 17	64	2	3.1
	Del 17	81	3	3.7
868	Del 18	66	2	3.0

Transformation efficiency was recorded as the percentage of PLBs that produced shoot primordia after 4 months of secondary selection on medium containing 30 mg L⁻¹ hygromycin and 30 mg L⁻¹ meropenem.

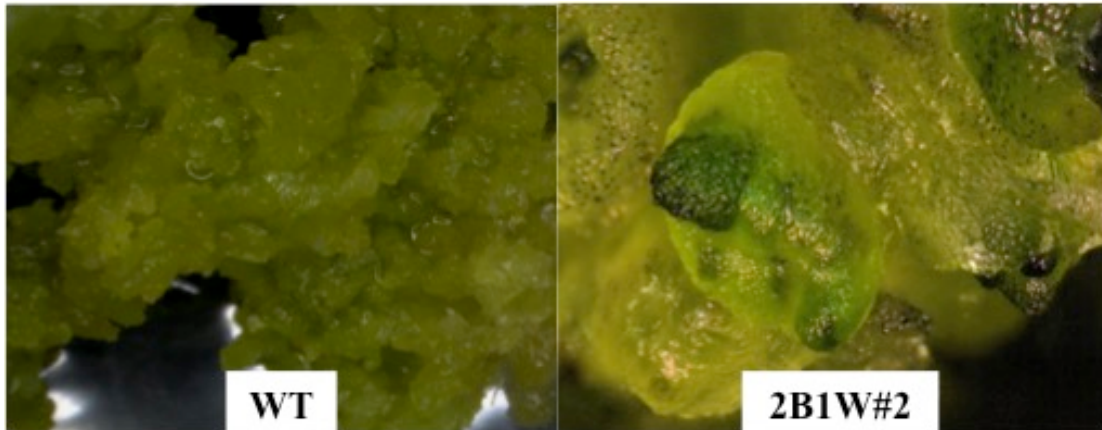


Figure 4-3. Expression of purple in hygromycin-resistant PLB tissue 'Watarun' × '868' of PLBs regenerated from independent protocorm inoculated with EHA101/pBIH-35D17-PDB after selected on selection medium containing 30 mg L⁻¹ hygromycin and 20 mg L⁻¹ meropenem for 2 years. These PLBs could be observed the purple color in tissue by microscope while could not be observed in control.

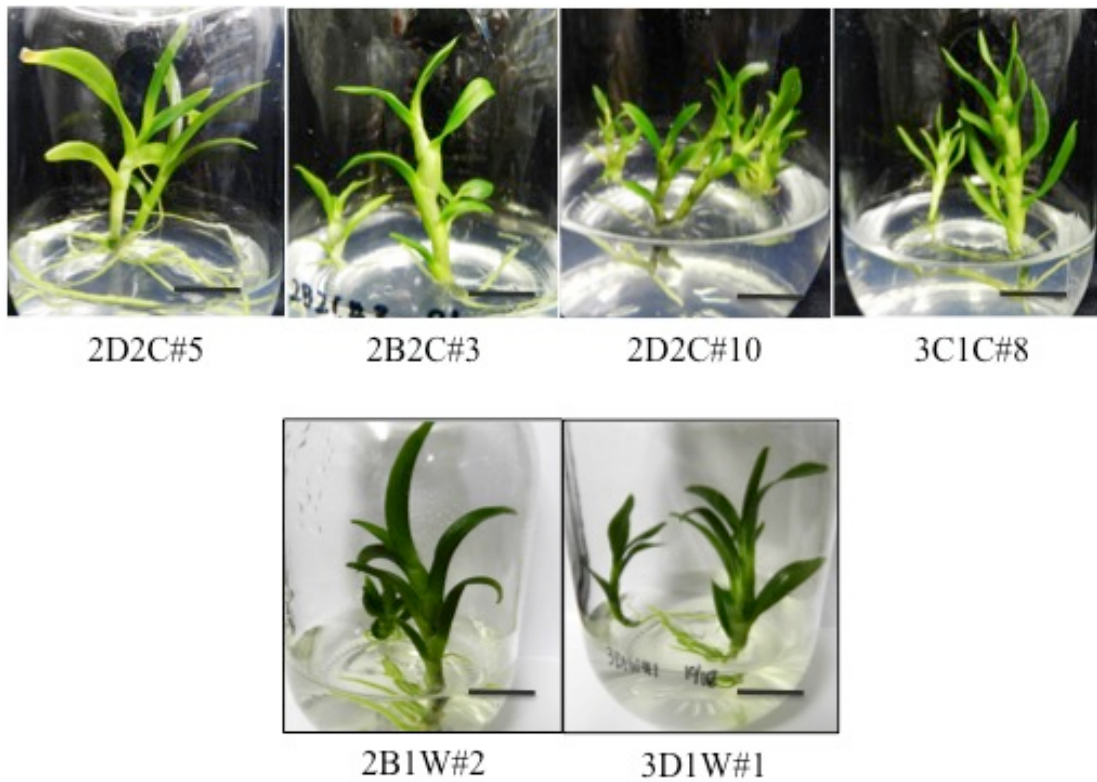


Figure 4-4. Regeneration and growth of transgenic *Dendrobium nobile* plantlets with different vectors

The regenerated plantlets of ‘Cinderella’ × ‘Truelove’ (Upper) and ‘Watarun’ × ‘868’ (Lower) from PLBs independent from 1 protocorm after selection on ND medium containing 30 mg L⁻¹ meropenem and 20 mg L⁻¹ hygromycin for 16 months.

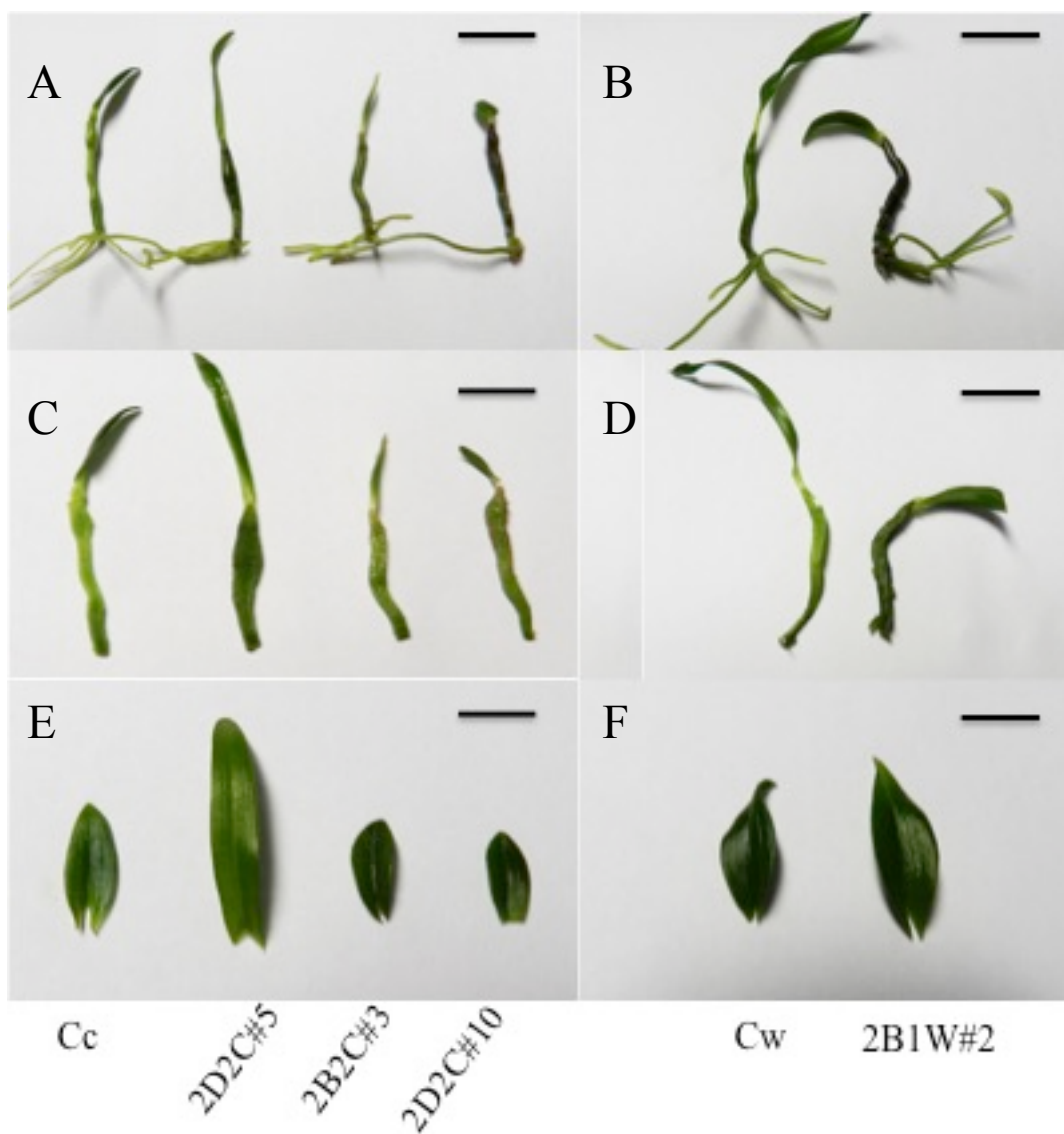


Figure 4-5. Expression purple in shoot and leaf transgenic plantlets inoculated with EHA101/pBIH-35D17-PDB.

The stems after peeling out the outer leaf sheath (A and B) cut longitudinal of stem (C and D), and leaves (E and F): ‘Cinderella’ × ‘Truelove’, (B, C, F): ‘Watrun’ × ‘868’.

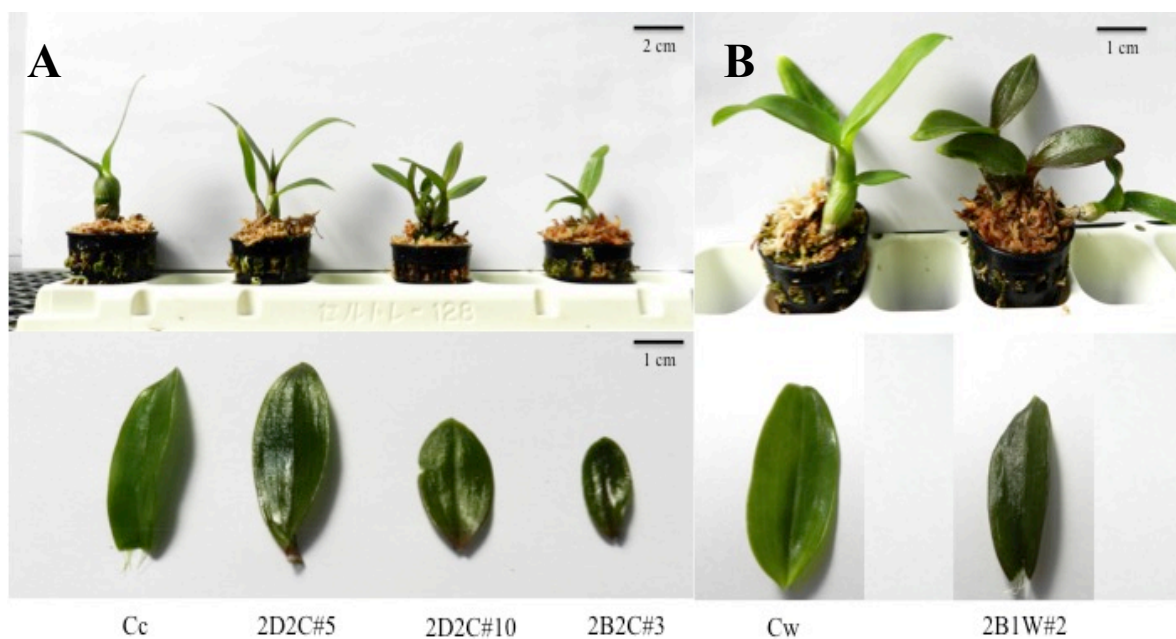


Figure 4-6. Acclimatized transgenic plants.

The transgenic plants were cultured in bottle containing 10 mg L^{-1} hygromycin and 10 mg L^{-1} meropenem in ND medium after growth until showing vigor shoot and leaves with roots. The plants were acclimatized with sphagnum moss into a plastic pot and cultured in the transgenic cultural greenhouse. (A: ‘Cinderella’ \times ‘Truelove’ and B: ‘Watrun’ \times ‘868’)

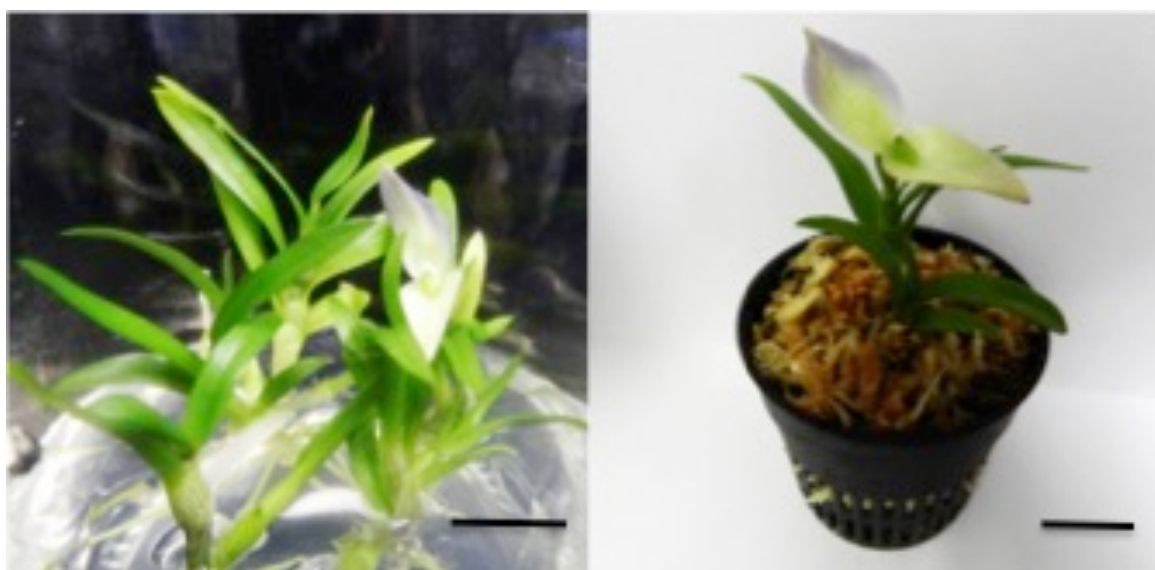


Figure 4-7. Production of blue flower-like structure in a transgenic plant of *D. nobile* ‘Cinderella’ × ‘Truelove’.

The transgenic plants were cultured in bottle containing 10 mg L^{-1} hygromycin and 10 mg L^{-1} meropenem ND medium for 2 years and sub-cultured every 4 months. These blue flowers were bloomed with deformed flower, but displaying with the blue color.

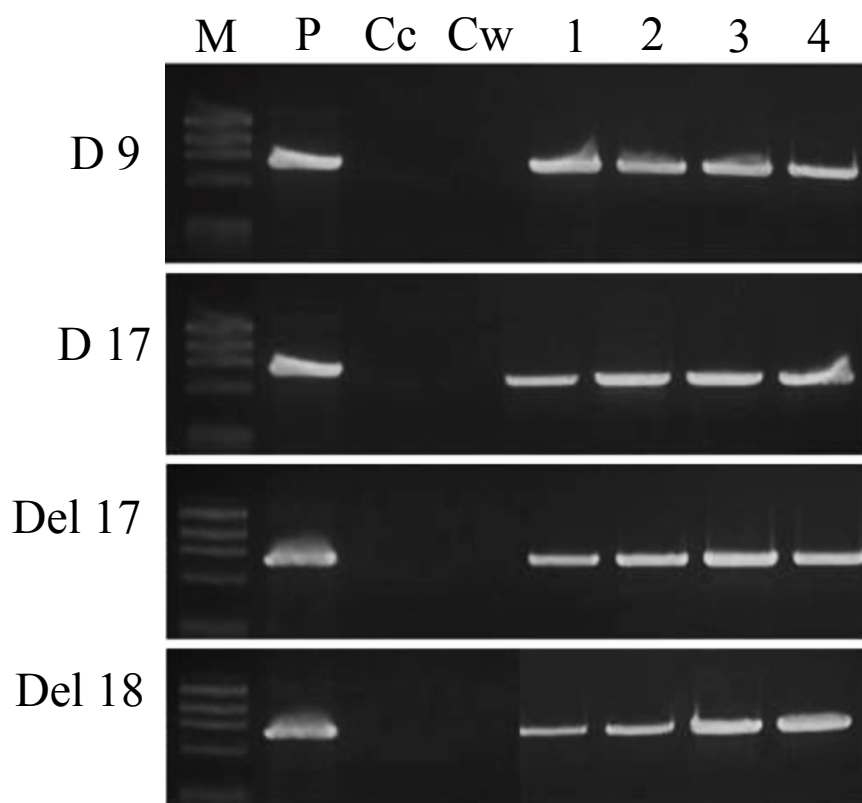


Figure 4-8. PCR analysis for *hpt* gene in putative transgenic plants derived from *Agrobacterium* - mediated transformation with different vectors.

(A: pBIH-35D9-PDB, B: pBIH-35D17-PDB, C: pBIH-35S-Del17, D: pBIH-35S-Del18)

Lane M: Molecular size marker (ϕ X174/*Hae*III)

Lane P: Plasmid (pBIH-35D9-PDB: A and B), (pBIH-35S-Del17: C and D)

Lane C: Non-transformed plants

(Cc: 'Cinderella' × 'Truelove', Cw: 'Watarun' × '868')

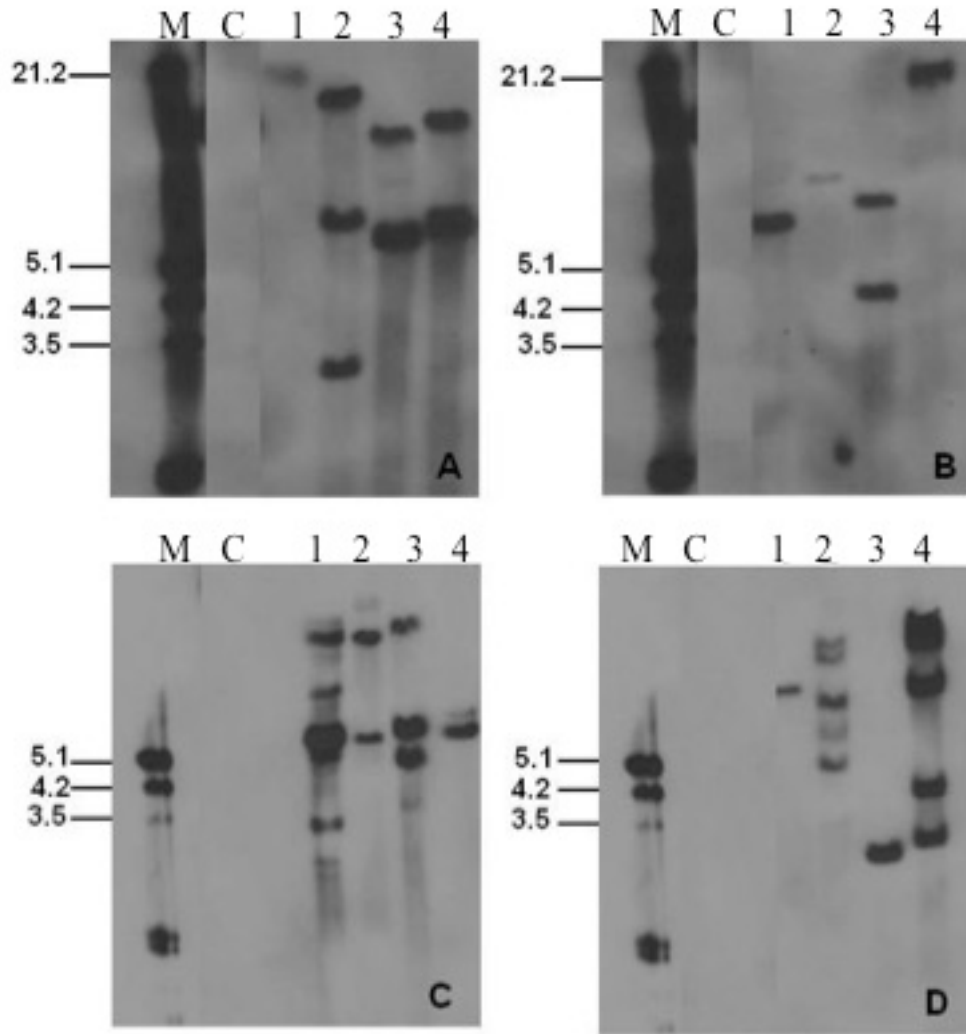


Figure 4-9. Southern blot analyses of transgenic plants

(A: pBIH-35D9-PDB, B: pBIH-35D17-PDB, C: pBIH-35S-Del17, D: pBIH-35S-Del18)

Lane M: Molecular size marker (λ DNA/*Eco*RI, *Hind*III)

Lane C: Non-transformed plants

(Cc: ‘Cinderella’ \times ‘Truelove’, Cw: ‘Watarun’ \times ‘868’)

Lanes 1-4: Transgenic plants (1-2: ‘Cinderella’ \times ‘Truelove’, 3-4: ‘Watarun’ \times ‘868’)

Probe: *hpt*

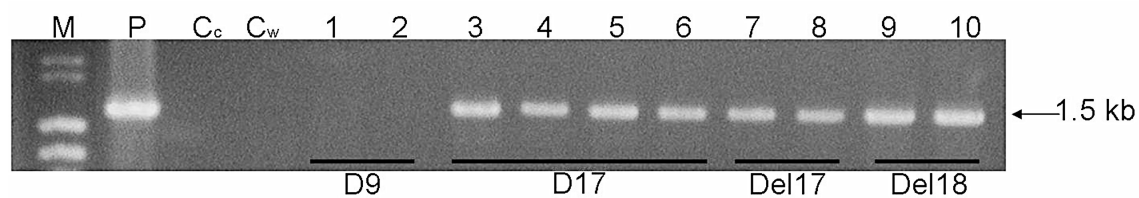


Figure 4-10. PCR analyses for F3'5'H gene in transgenic plants derived from *Agrobacterium* -mediated transformation with different vectors.

Lane M: Molecular size marker (ϕ X174/*Hae*III)

Lane P: Plasmid F3'5'H gene

Lane C: Non-transformed plants

(Cc: 'Cinderella' × 'Truelove', Cw: 'Watarun' × '868')

Lanes 1-10: Transgenic plants

(1,3,4,7,9: 'Cinderella' × 'Truelove', 'Watarun' × '868': 2,5,6,8,10:)

Remark; Lane 4 and 6: the purple shoot transgenic plants

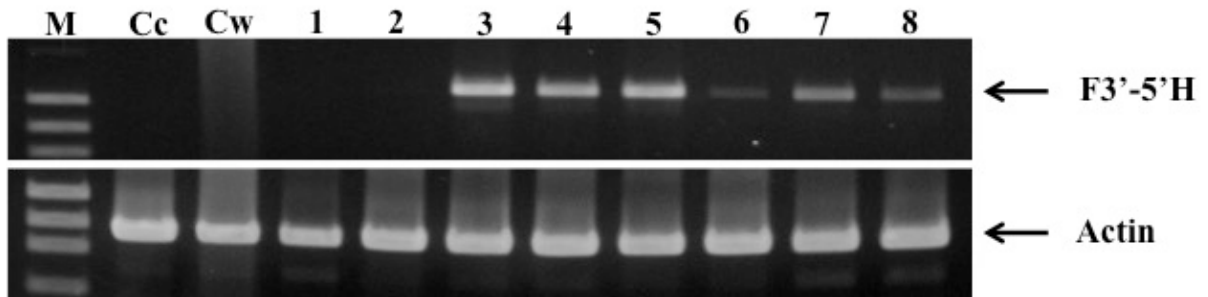


Figure 4-11. Expression of the *F3'5'H* gene.

RT-PCR fragment amplified from transgenic plants. Rice actin (RAc1) gene was used as an internal control for RNA input.

Lane M: Molecular size marker (λ /*Hind*III, ϕ X174/*Hae*III)

Lane C: Non-transformed plants

(Cc: 'Cinderella' \times 'Truelove', Cw: 'Watarun' \times '868')

Lanes 1-8: Transgenic plants

1, 2, 3, 4, 6: 'Cinderella' \times 'Truelove'

5, 7, 8: 'Watarun' \times '868'

Chapter 5

General Discussion and Conclusion

Part 5: General Discussion and Conclusion

General Discussion and Conclusion

Recent advances in genetic engineering have done the transformation and regeneration of plants as a powerful tool for orchid improvement. A simple and reproducible *Agrobacterium*-mediated transformation protocol is an extremely important method for transformation. Usually, the target tissues for gene transfer were used widely as protocorms and protocorm-like bodies (PLBs), were successfully introduced into constructed of foreign genes. In this study, efficient genetic transformation in *Dendrobium* was performed by using two target materials for the introduction of a target gene to produce transgenic *D. nobile* and *D. Formidible*, with a novel trait of blue. It is well-know that protocorms and protocorm-like bodies are important for propagation, and they are used on a large scale for commercial tissue culture.

Investigations were presented in Chapter 2 to discover the optimum transformation system, inoculation time, and bacteria concentration. This study utilized divergent genetic background as target material. A bacteria concentration of 10 times dilution from $OD_{600}=1.0$ gave better transformation efficiency than 50 times dilution. Similarly, results for other genera such as *Cymbidium*, *Vanda*, and *Cattleya* showed the success in the

transformation procedure obtained from high bacteria concentration. These results indicated that high bacteria concentration was also optimal for *Dendrobium*. Longer inoculation time of 300 min resulted in a higher extreme transformation efficiency than the shorter period of 30 min. An experiment which used protocorms as target material by Mishiba et al. (2005) was successful in producing transgenic plants after 7 h of inoculation time. This study indicated that an extended period of more than 5 h stimulated high transformation efficiency. Transformation efficiency of 27.3% was achieved with an inoculation time of 300 min and 10 times dilution of the bacteria concentration. Bacteria overgrowth covered the cocultivation medium after three days. The infected protocorms were washed with liquid new Dogashima medium (NDM) and transferred immediately to eliminate bacteria during selection. When covered by bacterial overgrowth they could not be properly seen. The overgrowth of bacteria can affect and destroy explants. The ability of meropenem was demonstrated to suppress the bacteria growth during the selection period. The optimum cocultivation period is important for genes introduced into the host plant for the success of the transformation system (Gnasekaran et al. 2014; Joyce et al. 2010; Yenchon et al. 2012). Molecular analysis was performed for integration in hygromycin (Hm) resistance to confirm the existence of the transgenic plants. This transformation procedure could be adopted for use in other cultivars, and the high transformation efficiency suggests the ability to obtain several transgenic plants from one experiment.

In Chapter 3 *Agrobacterium*-mediated transformation was discussed using PLBs as the target material. The most optimum conditions are good for gene transfer in a transformation system. Several important factors such as inoculation time, bacteria concentration, and incubation with or without rotary shaking were studied. The PLBs were investigated after cell dividing and activation by the inoculation medium. A 1:10 dilution of bacteria concentration and 30 min inoculation time with rotary shaking showed the highest transformation. Most cases of *Dendrobium* transformation with a short period of immersion in *Agrobacterium* suspension gave optimum conditions (Men et al., 2003; Yu et al., 2001). The target material must be susceptible to *Agrobacterium* suspension and easy to recover. PLBs after subculture for three weeks gave the highest transformation efficiency; this dropped sharply for period lengths more or less than this stage. The third week PLBs inoculated in only the carbon inoculation medium showed superior transformation efficiency of up to 18.5%. This did not change dramatically with liquid NDM, whereas it changed significantly using distilled water. These results indicated that the PLBs, after subculture for three weeks were at the optimum stage for stimulating cell dividing; only carbon (maltose) inoculation medium affected the transformation system. However, with or without ND composition liquid medium also contained 10 mg L⁻¹ maltose, but without ND medium was showed appropriate or susceptible for *Agrobacterium*. Nevertheless, liquid medium without ND composition adding 10 mg L⁻¹

maltose was suggested as the inoculation medium. The GUS assay system showed staining blue color from the PLB stage to plantlets. Hygromycin-resistance was confirmed to be transgenic by PCR and Southern blot analyses. Proliferated PLBs could be regenerated normally morphologically. Therefore, this efficient transformation system can be utilize to *Dendrobium* with a target gene to process a novel trait.

Chapter 4 investigated *D. nobile*, a potted plant with high demand in the floricultural ornamental market. *Dendrobium* lacks the violet and blue flower color because of the absence of delphinidin-based anthocyanins as the flavonoid-3', 5'-hydroxylase (*F3'5'H*), which is a key enzyme and essential for Delphinidin synthesis in anthocyanin biosynthesis. In this study, four different vectors harbored the *F3'5'H* gene isolated from *Commelina communis* and hygromycin transphotransferase (*hpt*) as selectable. The transformation procedure was described in Chapter 2. Using of four vectors, the integration of this target gene could be efficiently selected by PCR and Southern blot analyses technique with *hpt* primer and probe, respectively. Unfortunately, only three vectors D17, Del 17, and Del 18 could be confirmed in the *F3'5'H* gene as genomic by PCR analysis. Transcription levels also confirmed that three vectors could be performed by RT-PCR technique. Only the D 17 vector showed the purple shoots and leaves for the morphological transgenic plants. Three lines from 'Cinderella' × 'Truelove' showed the difference in morphological size and leaf patterns of small size like a dwarf. One line showed blue flowering, but deformed

with only sepals. All the transgenics showed purple shoots and leaves, not blooms. It was expected that the *in vivo* transgenic plants should express more intense blue than *in vitro* plants, because the shoots and leaves observed *in vivo* were deeper than those *in vitro*. The objective was to use protocorms derived from elite clone crossing. Different patterns and colors can be obtained, and the best patterns re-selected. The best transgenic line could be used for further commercial propagation.

This study established a transformation procedure using protocorms as target materials to obtain the highest transformation efficiency in *Dendrobium*. The objective was successful for application on a large scale in one experiment to support the generation of several elite plants. This efficient transformation procedure using PLBs was developed for producing the elite cultivars. The optimum stage of PLBs and the inoculation medium that gave the best conditions was applied to this genus. The transformation procedure in the protocorms for the production of blue *D. nobile* utilized the introduction of the *F3'5'H* gene. PLBs of *D. Formidible* and *D. phalaenopsis* exhibited transformation to the blue flower and also showed other novel traits, such as disease resistance and longer vase life.

References

References

- Arifin NS, Terauchi T, Tamura Y, Tanio M, Matsuoka M (2004) Effectiveness of sonication on diminishing bacterial overgrowth in *Agrobacterium*-mediated transformation. *Plant Biotechnol* 21: 53-56
- Azadi P, Chin DP, Kuroda K, Khan RS, Mii M (2010) Macro elements in inoculation and co-cultivation medium strongly affect the efficiency of *Agrobacterium*-mediated transformation in *Lilium*. *Plant Cell Tiss Organ Cult* 101: 201-209
- Bayer E, Egeter H, Fink A, Nether K. and Wegmann K, 1966. *Angew. Chem* 78, 834–841
- Belarmino MM, Mii M (2000) *Agrobacterium*-mediated genetic transformation of a phalaenopsis orchid. *Plant Cell Rep* 19: 435-442
- Brugliera F, Tao G Q, Tems U, Kalc G, Mouradova E, Price K, Stevenson K, Nakamura N, Stacey I, Katsumoto Y, Tanaka Y, Mason J G (2013) Violet/Blue chrysanthemums metabolic engineering of the anthocyanin biosynthetic pathway results in novel petal colors. *Plant Cell Physiol* 54(10): 1696-1710
- 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. Hort* 96: 213-224
- Chang C, Chen YC, Hsu YH, Wu JT, Hu CC, Chang WC, Lin NS (2005) Transgenic resistance to *Cymbidium mosaic virus* in *Dendrobium* expressing the viral capsid protein gene. *Transgenic Res* 14: 41-46
- Chandran S, Toh C L, Zulina R, Yip Y K, Nair H, Boyce A N (2006) Effects of sugars and aminoxyacetic acid on the longevity of pollinated *Dendrobium* (Heang Beauth) flowers. *J. applied Hort* 8(2): 117-120
- Cheng M, Lowe B A, Spencer T M, Ye X, Armstrong C L (2004). Factors influencing *Agrobacterium*-mediated transformation monocotyledonous species. *In Vitro Cellular*

and Development Biology 40:31-45

Chia TF, Chan YS, Chua NH (1994) The firefly luciferase gene as a non-invasive reporter for *Dendrobium* transformation. *Plant J* 6: 441-446

Chen L, Hatano T, Niimi Y (2002) High efficiency of *Agrobacterium*-mediated rhizome transformation in *Cymbidium*. *Lindleyana* 17: 130-134

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

Cogan N, Harvey E, Robinson H, Lynn J, Pink D, Newbury H J, Puddephat I (2001) The effects of anther culture and plant genetic background on *Agrobacterium rhizogenes*-mediated transformation of commercial cultivars and derived doubled-haploid *Brassica oleracea*. *Plant Cell Rep* 20: 755-762

Faria R T, Illg R D Propagação clonal de híbridos de *Dendrobium nobile* Lindl In: Congresso brasileiro de floricultura e plantas ornamentais, 10, 1995, Campinas. *Anais*. Campinas: ED. SBF. 40 - 41

Fukui Y, Tanaka Y, Kasumi T, Iwashita T, Nomoto K (2003) A rationale for the shift in colour towards blue in transgenic carnation flowers expressing the flavored 3'-5'-hydroxylase gene. *Phytochemistry* 63(1): 15-23

Guo W L, Chang Y C, Kao C Y (2010) Protocorm-like bodies initiation from root tips of *Cyrtopodium paranaense* (Orchidaceae). *Hort Sci* 45: 1365-1368

Gupta A, Pal R K, Rajam M V (2013) Delayed ripening and improved fruit processing quality in tomato by RNAi-mediated silencing three homologous of 1-aminopropane-1-carboxylate synthesis gene. *J. plant physiol* 170: 987-95

Hamill J D, Rounsley S, Spencer A, Todd G, Rhodes M J C (1991) The use of the polymerase chain reaction in plant transformation studies. *Plant Cell Rep* 10: 221-224

- Hayashi K, Abe Y, Mitsui S (1958). *Proc. Jpn. Acad.* 34, 373–378
- Hew C S (1994) Orchid cut-flower production in ASEAN countries. In *Orchid Biology: Reviews and perspective*, Vol. 6, ed. J. Arditti (John Wiley and Son Inc, New York) pp. 363-401
- Hood E E, Helmer G L, Fraley R T, Chilton M D (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J Bacteriol* 168: 1291-1301
- Hu J S, Ferreira S, Wang M, Xu M Q (1993) Detection of Cymbidium mosaic virus, Odontoglossum ringspot virus, tomato spotted wilt virus, and pot viruses infecting orchids in Hawaii. *Plant Dis* 77:464-468
- Ishii M (1968) Orchid Viruses and their control by chemical disinfection of nursery tools. *Bull. Pac. Orchid Soc. Hawaii* 26: 3-7
- Jefferson R A (1987) Assaying chimeric gene in plants: the GUS gene fusion system. *Plant Mol Biol Rep* 5: 387-405
- Julkifle A L, Poobathy R, Samian R, Subramaniam S (2012) Histological analyses of PLBs of *Dendrobium sonia-28* in the recognition of cell competence for regeneration and *Agrobacterium* infection. *Plant Omics J* 5: 514-517
- Kamemoto H, Amore T D, A Kuehnle R (1999) *Breeding Dendrobium Orchids in Hawaii. University of Hawaii Press, Honolulu*: 166
- Katsumoto Y, Fukuchi-Mizutani M, Fukui Y, Brugliera F, Holton TA, Karan M, Nakamura N, Yonekura-Sakakibara K, Togami J, Pigeaire A, Tao G Q, Nehra N S, Lu CY, Dyson B K, Tsuda S, Ashikari T, Kusumi T, Mason JG and Tanaka Y (2007). Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. *Plant Cell Physiol.* 48: 1589-1600
- Kondo T, Yoshida K, Nakagawa A, Kawai T, Tamura H, Goto T (1992) *Nature* 358, 515–518

- Kuehnle AR, Sugii N (1992) Transformation of *Dendrobium* orchid using particle bombardment of protocorms. *Plant Cell Rep* 11: 484-488
- Kuehnle A R (1997). Molecular biology of orchids. Orchid Biology: Reviews and Perspectives, VII. Kluwer Academic Publishers, Lancaster, England, *J. Arditti and A. Pridgeon*: 76-116
- Kuehnle A R, Amore T D, Mersino E, Sewake K, Wagoner T (2003) What do *Dendrobium* Orchid producers want in their potted flowers -results of a grower survey. *New plant for Hawaii*, july 2003: NPH-8
- Lekawatana S (2010). Thai Orchid: current situation. 2010 Taiwan International Orchids Symposium. March 5, 2010
- Liau C H, You S J, Prasad V, Hsiao H H, Lu J C, Yang N S, Chan M T (2003) *Agrobacterium tumefaciens*-mediated transformation of an *Oncidium* orchid. *Plant Cell Rep* 21: 993-998
- Martin KP, Geevarghese J, Joseph D, Madassery J (2005) In vitro propagation of *Dendrobium* hybrids using flower stalk node explants. *Indian J Exp Biol* 43: 280-285
- Men S, Ming X, Wang Y, Liu R, Wei C, Li Y (2003a) Genetic transformation of two species of orchid by biolistic bombardment. *Plant Cell Rep* 21: 592-598
- Men S, Ming X, Liu R, Wei C, Li Y (2003) *Agrobacterium*-mediated genetic transformation of a *Dendrobium* orchid. *Plant Cell Tiss Organ Cult* 75: 63-71
- Mishiba K, Chin D P, Mii M (2005) *Agrobacterium*-mediated transformation of *Phalaenopsis* by targeting protocorms at an early stage after germination. *Plant Cell Rep* 24: 297-303
- Murray M G, Thompson W F (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* 8: 4321-4326

- Nan G L, Kuehnle A R, Kado C I (1998) Transgenic *Dendrobium* orchid through *Agrobacterium*-mediated transformation. *Malayan Orchid Rev* 32: 93-96
- Noda N, Aida R, Kishimoto S, Ishiguro K, Mizutani M K, Tanaka Y, Ohmiya A (2013) Genetic engineering of novel bluer-colored chrysanthemums produced by accumulation of delphinidin-based anthocyanins. *Plant Cell Physiol.* 54 (10): 1684-1695
- Ogawa Y, Mii M (2004) Screening for highly active β -lactam antibiotics against *Agrobacterium tumefaciens*. *Arch Microbiol* 181: 331-336
- Ogawa Y, Mii M (2007) Meropenem and moxalactam: novel β -lactam antibiotics for efficient *Agrobacterium*-mediated transformation. *Plant Sci* 172: 564-572
- Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a β -glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol* 31: 805-813
- Rasika G M J, Barkhurst H L, Amore T D (2013) Predominance of purple color in *Dendrobium* is due to enzyme competition between DFR and F3'H. Methods for Expanding Color Palette in *Dendrobium* Orchids. *Justia patents: Application #20140201865*
- Park C H, Chae S C, Park S Y, Kim J K, Kim Y J, Chung S O, Arasu M V, Al-dhabi N B, Park S U (2015) Anthocyanin and caroteneoid contents in different cultivars of *Chrysanthemum* (*Dendrathera grandiflorum* Ramat.) flower. *Molecules*. ISSN 1420-3049
- Pimda W, Bunnag S (2010). Protocorm wounding enhanced *Agrobacterium*-mediated transformation of *Hygrochilus parishii* (Veitch & Rchb.f.) Pfitz. *Advances in Agriculture & Botany International Journal of the Bioflux Soci.* 2: 205-212
- Puchooa D (2004) Comparison of different culture media for the in vitro culture of *Dendrobium* (Orchidaceae). *Int. J. Agric. Biology* 6: 884-888

- Shimoda N, Toyoda-Yamamoto A, Nagamine J, Usami S, Katayama M, Sakagami Y, Machida Y (1990) Control of expression of *Agrobacterium vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. *Proc Natl Acad Sci USA* 87: 6684-6688
- Shiono M, Matsugaki N, and Takeda K, 2008. Structure of commelinin, a blue complex pigment from the blue flowers of *Commelina communis*. *Proc Jpn Acad Ser B Phys Biol Sci.* 84 (10): 452-456
- Shrestha B R, Chin D P, Tokuhara K, Mii, M (2007) Efficient production of transgenic plants of *Vanda* through sonication-assisted *Agrobacterium*-mediated transformation of protocorm-like bodies. *Plant Biotech* 24: 429-434
- Sjahril R, Mii M (2006) High-efficiency *Agrobacterium*-mediated transformation of *Phalaenopsis* using meropenem, a novel antibiotic to eliminate *Agrobacterium*. *J. Hortic. Sci. Biotechnol* 81: 458-464
- Sood P, Bhattacharya A, Sood A (2011) Problems and possibilities of monocot transformation. *Biologia Plantarum* 55: 1-15
- Suwanaketchanatit C, Piluek J, Peyachoknagul S, Huehne PS (2007) High efficiency of stable genetic transformation in *Dendrobium* via microprojectile bombardment. *Biol Plant* 51: 720-727
- Tee C S, Marziah M, Tan C S, Abdullah M P (2003) Evaluation of different promoters driving the GFP reporter gene and selected target tissues for particle bombardment of *Dendrobium Sonia* 17. *Plant Cell Rep* 21: 452-458
- Tokuhara K, Mii M (1993) Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. *Plant Cell Rep* 13: 7-11
- Wise A A, Voinov L, Binns A N (2005) Intersubunit complementation of sugar signal transduction in *VirA* heterodimers and posttranslational regulation of *VirA* activity in *Agrobacterium tumefaciens*. *J Bacteriol* 187: 213-223

- Yamada T, Takatsu Y, Manabe T, Kasumi M, Marubashi W (2003) Suppressive effect of trehalose on apoptotic cell death leading to petal senescence in ethylene-insensitive flowers of gladiolus. *Plant Science* 164: 213-221
- Yu H, Yang SH, Goh CJ (2001) *Agrobacterium*-mediated transformation of a *Dendrobium* orchid with the class 1 *knox* gene *DOH1*. *Plant Cell Rep* 20: 301-305
- Xiao L, Ha S B (1997) Efficient selection and regeneration of creeping bentgrass transformants following particle bombardment. *Plant Cell Rep* 16: 874-878
- Zhang L, Chin D P, Mii M (2010) *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cattleya*. *Plant Cell Tissue Organ Cult* 103: 41-47
- Zheng Q, Zheng Y P, Wang G D, Guo W M, Fan E F, Wang C (2012) Sonication assisted *Agrobacterium*-mediated transformation of *ACC* gene to interfere the production of ethylene in Spring *Dendrobium* cv. 'Sanya'. *Russ J Plant physiol* 59: 266-274

Appendix

Appendix 1. Composition of New Dogashima Medium (NDM) contents used in the present study (Tokuhara and Mii, 1993)

	(mg L ⁻¹)
Macro Nutrients	
KNO ₃	202.0
KH ₄ NO ₃	481.0
KH ₄ PO ₄	554.0
KCl	152.0
Ca(NO ₃).4H ₂ O	470.0
MgSO ₄ .7H ₂ O	250.0
Fe-EDTA	42.0
Micro Nutrients	
MnSO ₄ .4H ₂ O	3.0
ZnSO ₄ .7H ₂ O	0.5
H ₃ BO ₃	0.5
Na ₂ MoO ₄ .2H ₂ O	0.025
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Concentrated H ₂ SO ₄ (μl/l)	0.5
Vitamins	
Myo-Inositol	100.0
Nicotinic acid	1.0
L-Cysteine	1.0
Thiamine hydrochloride	1.0
Pyridoxine hydrochloride	1.0
Adenine	1.0
Calcium (+)-Pantothenate	1.0
(+)-Biotin	0.1

pH 5.4

Appendix 2. Composition of LB medium contents used in the present study

	(g L ⁻¹)
Tryptone peptone (DIFCO)	10.0
Yeast extract (DIFCO)	5.0
NaCl (WAKO)	10.0
Agar powder (WAKO)	15.0

pH 7.2

Appendix 3. Composition of reaction solutions used for GUS assay

Stock solution	Composition	Amount
1. 0.5 M Phosphate buffer H ₂ O	NaH ₂ PO ₄ ·2H ₂ O	7.8 g/50 ml
		pH 7.0
2. 10 ml Lysis buffer stock	0.5 M NaH ₂ PO ₄ ·2H ₂ O	1 ml
	20 % Methanol	2 ml
	0.5 % β-melchaphthoethanol	50 μl
	Distilled Water	7 ml
3. 1 mM X-Gluc stock	X-Gluc	20 mg
	Dimethylformamide	1 ml

X-Gluc = 5-bromo-4chloro-3-indolyl-β-D-glucuronic acid

Store solution number 1, at room temperature; solution number 2, and 3 at -20°C

For a method *GUS* assay:

1.0 ml of 50 mM phosphate buffer stock solution (2) is mixed with 26.0 μl of X-Gluc stock solution (3) and dispensed evenly into tubes containing samples to be analyzed, sealed, air-vacuumed for 15 min and incubated in 37 °C at overnight.

Appendix4. Chemical compositions of CTAB method used in the present study
(200 ml solution)

2% CTAB Solution	Amount
Tris-HCL (pH 8.0)	100.0 mM
EDTA (pH 8.0)	20.0 mM
NaCl	1.4 M
CTAB	4.0 g
1% CTAB Solution	
Tris-HCL (pH 8.0)	50.0 mM
EDTA (pH 8.0)	10.0 mM
CTAB	2.0 g
TE Buffer	
Tris-HCL (pH 8.0)	10.0 mM
EDTA (pH 8.0)	1.0 mM

Appendix 5. Composition of reaction solutions used for PCR analysis

	Amount
DNA sample	1.0 μ l
10X EX-Taq buffer	2.0 μ l
dNTP Mix (2.5 mmol)	2.5 μ l
Forward Primer (50 pmol/100 μ l)	0.5 μ l
Reverse Primer (50 pmol/100 μ l)	0.5 μ l
Taq DNA polymerase (5 unit/ml)	0.1 μ l
Distilled Water	18.4 μ l

Total	25.0 μ l
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Summary

Summary

Dendrobium is one of the largest genera in the family and the most popular orchids grown for commercial production of cut flower and pot plants, the commonly cultivated species include: *D. phalaenopsis*, *D. bigibbum* and *D. nobile*. Recently, cultivars with novel flower colors, shapes and disease resistance have been developed through intraspecific and interspecific hybridizations. Among these, *D. nobile* is representative as a famous potted plant, which differentiates itself from the other orchid by a cluster of flowers that opposite the axil of each leaf. Although it has large variations in flower color, however it lacks blue to violet cultivars. *D. Formidible* is interspecific hybrid between *D. formosum* and *D. infundibulum* which Most cultivars of *D. Formidible* have large white flowers (ca. 12 cm in diameter) with yellow eye and usually bloom during early to mid summer season. However, lacks color variations and novel cultivars with other flower colors are now expected because of difficulty in crossing with other *Dendrobium* species and cultivars. In this study, I established efficient *Agrobacterium*-mediated genetic transformation systems using target from protocorm and protocorm-like body for genetic improvement in *Dendrobium*. Then, production of blue-purple flowering *D. nobile* derived from delphinidin accumulating by introducing *Commelina communis* F3'5'H gene was

obtained.

The contents of this thesis are consisted of 5 chapters. The background of this study and *Agrobacterium*-mediated gene transfer was provided in Chapter 1. The results obtained using the present study summarized below as Chapters 2-4. Finally, discussion and conclusion were achieved based on these results in Chapter 5.

Chapter 2: High efficiency *Agrobacterium*-mediated transformation of *Dendrobium* orchid using protocorms as a target material

Dendrobium orchid is one of the most popular cut flower and potted plants. In this study, a protocol for efficient genetic transformation of *D. nobile* was established by co-cultivating 21 day-old protocorms for 3 days with *Agrobacterium tumefaciens* strain EHA101 carrying pIG121Hm harboring β -glucuronidase (*GUS*) gene as reporter gene and hygromycin phosphotransferase (*hpt*) gene as selectable marker gene. After selection of the infected protocorms on New Dogashima (ND) medium containing 10 g L⁻¹ maltose, 30 mg L⁻¹ hygromycin and 20 mg L⁻¹ meropenem for 3 months followed by the culture on hygromycin-free recovery medium for 1 month, secondary protocorm-like bodies (PLBs) produced on this medium were again transferred onto secondary selection (regeneration) medium. Plantlets were successfully regenerated from these secondary PLBs after the

transfer. The highest transformation efficiency of 27.3% was obtained when protocorms were inoculated with 10 times diluted *Agrobacterium* solution ($OD_{600}=0.1$) for 300 minutes. Transformation of the selected plants was confirmed by GUS assay, PCR and Southern blot analysis. This protocol could be adopted to produce transgenic *D. nobile* orchids with various traits such as novel flower color and resistances to biotic and abiotic stresses.

Chapter 3: *Agrobacterium*-mediated transformation of protocorm-like bodies in

***Dendrobium* Formidible ‘Ugusu’**

Agrobacterium-mediated genetic transformation system was established in *Dendrobium* Formidible ‘Ugusu’ by inoculating PLBs with *A. tumefaciens* strain EHA101 (pIG121Hm) harboring hygromycin phosphotransferase (*hpt*) and neomycin phosphotransferase II (*nptII*) genes as selectable marker gene and β -glucuronidase (*gus*) gene as a reporter gene. For obtaining the optimum conditions for the transformation, several factors such as the stage of PLBs after subculture, bacterial concentrations, kind of inoculation medium, inoculation time and inoculation condition (with or without rotary shaking), were examined. After inoculation, PLBs were cocultivated for 3 days and then transferred for selection onto 2.5 g L⁻¹ gellan gum-solidified ND medium containing 10 g L⁻¹ maltose, 20 mg L⁻¹ hygromycin

and 20 mg L⁻¹ meropenem. Hygromycin-resistant plantlets were regenerated from secondary PLBs after 4 months of selection. Transformation of these plants was confirmed by GUS histochemical assay, PCR and Southern blot analyses. The highest transformation efficiency of 18.5% was obtained when PLBs 3 weeks after subculture were inoculated with 1:10 diluted bacteria (OD₆₀₀≈0.1) with liquid medium containing only 10 g L⁻¹ maltose and 100 μM acetosyringone with shaking for 30 min

Chapter 4: Blue gene expression in *D. nobile* by introducing flavonoid 3', 5'-hydroxylase gene via *Agrobacterium*-mediated transformation

Flavonoid-3', 5'-hydroxylase (*F3'5'H*) is a key enzyme in the synthesis of 3', 5'-hydroxylated anthocyanins, which are required for the expression of blue flower color. In this study, an efficient protocol for genetic transformation of *D.nobile* was established by cocultivating for 3 days, young protocorms (21 days after sowing) with *Agrobacterium tumefaciens* EHA101 harboring each of 4 vectors containing flavonoid-3', 5'-hydroxylase (*F3'5'H*) gene, for blue production of blue flower color. After selection of the inoculated protocorms on ND medium containing 1% maltose, 30 mg L⁻¹ hygromycin and 20 mg L⁻¹ meropenem for 4 months, regenerated plantlets were obtained from resistant protocorms through secondary protocorm-like body (PLB) proliferation. The efficiency of protocorm

with hygromycin resistant derived from independent protocorms was 3.1 – 8.8% and the regenerated plantlets, the shoots and leaves showed bluish coloration that bloomed purple like blue flower. Integration and expressing of the transgene in the transgenic plants were confirmed by PCR, RT-PCR and Southern blot analyses.

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