CHAPTER III

RESEARCH METHODOLOGY

Plant materials

Flowering *Dendrobium* Sonia cv. Earsakul plants were obtained from orchid farms in Nakhon Phathom and Ratchaburi provinces, Thailand. Flowers at seven different developmental stages, from early buds to fully-opened flowers, were collected for RNA extraction. Developmental stages are as follows; Stages 1 (< 2 cm), 2 (2.0-2.3 cm), 3 (2.8-3.0 cm), 4 (3.3-3.5 cm), 5 (3.8-4.0 cm), 6 (opening) and 7 (opened). The attached flower stages 2 and 3 were used for agroinfiltration.

Anthocyanin analysis

The anthocyanin was isolated from the flower buds of *D.* Sonia cv. Earsakul according to Kubo, et al. (1999) and Hung, et al. (2008). One gram of petals from flower buds was ground in an ice cold 100 mM potassium phosphate buffer containing 1mM ascorbic acid. Each sample was centrifuged at 22,000g for 30 min at 4°C. After centrifugation, the absorbance of supernatant was measured at 600 nm using a spectrophotometer (OPTIZEN 3220UV, Korea). One absorbance unit was defined as the amount of the substance giving an absorbance of 1.0 at 600 nm in a 1 ml cuvette. The amount of anthocyanin contents was calculated from10-fold diluted samples.

RNA extraction

Total RNA extraction from the sepals and petals of *D.* Sonia cv. Earsakul was performed according to Monmai and Ratanasut (2012). Up to 100 mg of fresh samples were powdered in liquid N_2 and cell lysis was performed with 650 µl of TLES buffer (0.1 M Tris-HCl pH 8.0, 0.1 M LiCl, 10 mM EDTA, 1% SDS, and 2% Na_2SO_3). Five hundred microliters of phenol was then added and mixed vigorously. Each sample was centrifuged at 13,000 rpm for 10 min. After centrifugation, the separated aqueous layer was transferred to a fresh microcentrifuge tube and extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and then centrifuged at 13,000 rpm for

10 min. The upper aqueous phase was transferred into a fresh microcentrifuge tube. Five hundred microliters of 10 M LiCl was added into the supernatant for precipitation and then incubated at 4°C overnight. Total RNA was precipitated by centrifugation at 13,000 rpm at room temperature for 10 min. The pellets were dissolved in 200 µl of RNase-free water. RNA suspension was added with 20 µl of 2.5 M CH_3COONa and 500 µl of absolute ethanol. The RNA was allowed to precipitate at -80°C for 60 min and recovered by centrifugation at 13,000 rpm for 10 min at 4°C. The RNA pellets were washed with 1 ml of 70% ethanol, air dried for 5-10 min, and resuspended in 20 µl of RNase-free water and stored at -80°C until needed.

Cloning of partial *DFR* cDNA from *D.* Sonia cv. Earsakul

1. RNA analysis

The purified total RNA was quantified using the Qubit®Fluorometer (Invitrogen, USA) according to the manufacturer's instructions. The integrity of the total RNA was analyzed on 1.5% (w/v) agarose TAE gel electrophoresis.

2. First strand cDNA synthesis

The total RNAs from *D.* Sonia cv. Earsakul were used as templates to generate first strand cDNA in a reverse transcription reaction. To eliminate DNA in RNA samples, total RNA (about 600 ng) was treated with RQ1 RNase–free Dnase (Promega, USA) following the manufacturer's instructions. Three microliters of Dnase-treated RNA were mixed with 20 ng/µl of random hexamer and incubated at 70°C for 5 min followed by quick chill on ice for 5 min. Total random hexamer-RNA mixture was incubated with 15 µl of reverse transcriptase mix containing 1X Improm-II™ reaction buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs and 1 unit of Improm-II™ Reverse-Transcriptase (Promega, USA) at 25°C for 5 min followed by incubation at 42°C for 60 min. The reverse transcription reaction was inactivated at 70°C for 15 min and stored at -20°C until needed.

3. Isolation of partial *DFR* cDNA by PCR

The primers used for *DFR* isolation were designed based on the nucleotide sequence of *DFR* mRNA of *Dendrobium* hybrid cultivar (accession no. FM209431)

Two microliters of cDNA were used as templates in PCR reaction containing 0.375 µM of forward primer (DFR-F2: 5'CAATCAACGGTCTGCTGG),

0.375 μM of reverse primer (DFR-R2: 5'CTGTGGAGTCATAGGAAG), 0.2 mM of dNTPs mix, 1.5 mM of $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Vivantis, Malaysia). The PCR cycling conditions were predenaturation at 92°C for 2 min, 35 cycles of 92°C for 30 sec, 50°C for 20 sec, 72°C for 30 sec, followed by a final extension at 72°C for 5 min. PCR products were analyzed on 1.2% (w/v) agarose TAE gel electrophoresis and visualized by ethidium bromide staining.

4. DNA extraction from agarose gels

Extraction of DNA from agarose gels was carried out using a HiYield™ Gel/PCR fragments Extraction Kit (RBC Bioscience, Taiwan) following the manufacturer's protocol. The purified DNA was determined on 1.2% (w/v) agarose TAE gel electrophoresis.

5. Cloning of PCR products

The purified PCR products were cloned using pGEM®-TEASY vector system (Promega, USA) following the manufacturer's instructions. The molar ratio used for ligation was 1:3 (vector:insert) and the ligation reaction was incubated at 4°C overnight.

6. Preparation of competent cells

A single colony of *Escherichia coli* strain DH5α was inoculated into 5 ml of LB broth (Pronadisa, Spain). The *E. coli* cells were cultured at 37°C with shaking (250 rpm) for 8-12 hr. One milliliter of the cell culture was transferred into 50 ml of fresh LB broth and incubated at 37°C with shaking (250 rpm) for 2-3 hr until OD600 reached 0.3-0.4. The cultured cells were transferred to 50 ml centrifugal tube and placed on ice for 10 min before centrifugation at 4,000 rpm, 4°C for 10 min. Cell pellets were washed with 10 ml of 0.1 M $CaCl_2$ (ice-cold) and incubated on ice for at least 10 min followed by centrifugation at 4,000 rpm, 4°C for 10 min. The cell pellets were resuspended in 2 ml of 0.1 M $CaCl_2$ (ice-cold) and sterile glycerol was added to a final concentration of 30% (v/v) and incubated on ice for 15 min. The competent cells were stored at -80°C as 200 μl aliquots in 0.5 ml microcentrifuge tubes.

7. The *E. coli* transformation

Five microliters of ligation products were gently mixed with 100 µl of *E. coli* strain DH5α competent cells. The mixture was left on ice for 10-15 min. The competent cells were then incubated at 42°C for exactly 45 sec and immediately chilled on ice for 2 min. One milliliter of LB medium were added and the transformed cells were incubated at 37°C for 60 min. The suspended cells were spread on LB agar plate containing 100 µg/ml ampicillin, 0.002% (w/v) X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and 0.02 mM IPTG (isopropyl beta-D-thiogalactopyranoside). At the end of 60 min, it was followed by centrifugation at 13,200 rpm for 30 sec, poured out of old LB broth and added 200µl of new LB broth. The transformants were allowed to grow at 37°C overnight and selected by blue/white colony selection and colony PCR.

Plasmid DNA extraction

A single bacterial colony was inoculated into 5 ml of LB medium containing appropriate antibiotics (100 µg/ml ampicillin, 25 µg/ml kanamycin or 25 µg/ml spectinomycin). Cell culture was incubated at 37°C overnight with shaking (250 rpm). Plasmid DNA was extracted from cultured bacterial cells using HiYield™ Plasmid Mini Kits (RBC Bioscience, Taiwan) following the manufacturer's protocol. The extracted DNA was stored at -20°C until used.

Sequence analysis of the *DFR* clones

The *DFR* clones were sequenced by the service of First BASE Laboratories Sdn Bhd, Malaysia. The sequences of the *DFR* clones were analyzed using blastn (www.ncbi.nlm.nih.gov/blast).

Cloning of the *DFR*-hairpin RNA binary vectors using Gateway Technology

1. Cloning *DFR* fragments into pENTR

pGEM®-TEASY vector containing *DFR* fragments of *D.* Sonia cv. Earsakul (pGEM-*DFR*) was digested with *Eco*R I (10-GO RE kit, Vivantis, Malaysia). The digested plasmids were electrophoresed on 1.2% (w/v) agarose gel and visualized by ethidium bromide staining. The corresponding *DFR* bands were purified from

agarose gels using a HiYield™ Gel/PCR fragments Extraction Kit (RBC Bioscience, Taiwan) following the manufacturer's protocol.

pENTR™ 3C Dual vector (Invitrogen, USA) was digested with *Eco*R I (10-GO RE kit, Vivantis, Malaysia) following the manufacturer's protocol. One unit of FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas, Canada) was added to the *Eco*R I digestion. The digested plasmids were electrophoresed on 1.2% (w/v) agarose TAE gel and visualized by ethidium bromide staining. The 2.3 kb pENTR without the ccdβ gene were purified from agarose gels using a HiYield™ Gel/PCR fragments Extraction Kit (RBC Bioscience, Taiwan) following the manufacturer's protocol. *DFR* fragments with *Eco*R I ends were ligated to pENTR with *Eco*R I ends using 3 units of T4 DNA ligase (Invitrogen, USA). The ligation reactions were incubated at 14°C for 1 hr followed by incubation at 4°C overnight.

2. Transformation of pENTR-*DFR* into *E. coli*

Five microliters of ligation products were gently mixed with 50 µl of *E. coli* strain DH5α competent cells. The mixture was left on ice for 30 min. The competent cells were then incubated at 42°C for exactly 45 sec and immediately chilled on ice for 2 min. One milliliter of LB medium were added and the transformed cells were incubated at 37°C for 60 min. The suspended cells were spread on the LB agar plate containing 25 µg/ml Kanamycin. The transformants were allowed to grow at 37°C overnight.

3. Analysis of pENTR-*DFR* clones

3.1 pENTR-*DFR* clone analysis by colony PCR

Selected colonies derived from the selective medium containing kanamycin were screened for *DFR* inserts by PCR. The pGEM-*DFR* clones and nuclease-free water were used as the positive and negative controls, respectively. The PCR products were electrophoresed on 1.2% (w/v) agarose TAE gel and visualized by ethidium bromide staining.

3.2 pENTR-*DFR* clone analysis by *Eco*R I digestion

The PCR positive colonies were cultured in 5 ml of LB medium containing 25 µg/ml kanamycin. The pENTR-*DFR* clones were digested with FastDigest® *Eco*R I (Fermentas, Canada). The *DFR* inserts were determined on 1.2% (w/v) agarose TAE gel electrophoresis.

4. Construction of *DFR*-hairpin RNA binary vectors

The *DFR*-hairpin RNA binary vector used for *DFR* suppression in *D.* Sonia cv. Earsakul flowers was pSTARGATE and pWATERGATE obtained from CSIRO Plant industry, Australia. Successful clones pENTR-*DFR* was used to generate pSTARGATE-*DFR* and pWATERGATE-*DFR* by the LR recombination technique. The LR recombination reaction contained 100 ng of pENTR-*DFR*, 150 ng of pSTARGATE or pWATERGATE, 1X of LR Clonase™ II enzyme mix (Invitrogen, USA), and TE buffer (pH 8.0) to a final volume of 10 µl. The reaction was incubated at 25°C for 1 hr followed by adding 1 µl of Proteinase K solution (2 µg/µl) and incubated at 37°C for 10 min to inactivate the enzyme.

5. Transformation of pSTARGATE-*DFR* and pWATERGATE-*DFR* into *E. coli*

Two microliters of pSTARGATE-*DFR* or pWATERGATE-*DFR* ligation products were gently mixed with 100 µl of *E. coli* DH5α competent cells. The mixture was left on ice for 10 min. The competent cells were then incubated at 42°C for exactly 45 sec and immediately chilled on ice for 2 min. One milliliter of LB medium were added and the transformed cells were incubated at 37°C for 60 min. The suspended cells were spread on the LB agar plate containing 25 µg/ml spectinomycin. The transformants were allowed to grow at 37°C overnight.

Semi-quantitative expression analysis of *DFR* gene in *D.* Sonia cv. Earsakul flowers

Analysis of *DFR* gene expression in *D.* Sonia cv. Earsakul flowers was performed by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The PCR recipe and condition for *DFR* gene amplification were as described above. Expression of the *actin* gene was determined as an internal control. The PCR reaction contained 0.375 µM forward primer 5'-TATTGTGCTTGATTCTGGTG (DenActinF1), 0.375 µM reverse primer 5'-AGTTGTATGTTGTCTCGTG (DenActinR1), 0.2 mM dNTPs mix, 1.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Vivantis, Malaysia) and 2 µl of cDNA. The PCR cycling conditions were predenaturation at 92 °C for 2 min, 28 cycles of 92 °C for 30 sec, 50 °C for 20 sec, 72 °C for 30 sec, followed by a final

extension at 72 °C for 5 min. PCR products were electrophoresed on 1.2% (w/v) agarose TAE gel and visualized by ethidium bromide staining.

Agrobacterium-based transient transformation of *D.* Sonia cv. Earsakul flowers

1. *Agrobacterium* transformation

pSTARGATE-*DFR* and pWATERGATE-*DFR* were mobilized into *A. tumefaciens* strain EHA105 through triparental mating with *E.coli* strain GJ23 containing the helper plasmids (Van, et al., 1983). *A. tumefaciens* strain EHA105 was cultured in 2 ml of LB medium with shaking at 28°C (250 rpm) for 48 hr. *E. coli* strain DH5α containing pSTARGATE-*DFR* and pWATERGATE-*DFR* was co-cultured with *E. coli* strain GJ23 in LB medium with shaking at 37°C for 24 hr. Two hundred microliters of DH5α and GJ23 co-culture was mixed with 100 µl of *A. tumefaciens* strain EHA105 in 1.5 ml microcentrifuge tube and allowed to stand at room temperature for 1 hr. The mixed cells were spread on an LB agar plate containing 20 µg/ml rifampicin and incubated at 28°C for 48 hr. After colonies formed, 2 ml of 10 mM $MgCl_2$ were added on the LB agar surface and smeared with a sterile spreader in an aseptic condition. The suspended cells were diluted into 1:1000, 1:10000 and 1:100000. One hundred microliters of each dilution were spread on LB agar plates containing 25 µg/ml spectinomycin and incubated at 28°C for 48 hr.

A single colony of *A. tumefaciens* strain EHA105 carrying pSTARGATE-*DFR* or pWATERGATE-*DFR* was cultured in 5 ml of LB medium containing 20 µg/ml rifampicin at 28°C for 48 hr with shaking (250 rpm). The cultured cells were refreshed by transferring 200 µl of the cell culture into 10 ml of LB medium containing 20 µg/ml rifampicin and allowed to grow at 28°C for 24 hr with shaking (250 rpm). The cultured cells were collected by centrifugation at 5,600 rpm for 3 min and washed twice by vortexing with 1 ml of Deionized water (DI water). The cell pellets were resuspended in 1 ml of DI water with 100 µM acetosyringone (AS) and were diluted with DI water until the OD_{600} was 0.5.

2. *Agrobacterium* infiltration (agroinfiltration) of *D.* Sonia cv. Earsakul flowers

A suspension of *Agrobacterium* as mentioned above was used to infiltrate flowers of *D.* Sonia cv. Earsakul at different developmental stages attached to the plant

by a 1 ml syringe. The syringe with needle was firstly used to pierce sepals or petals. The volume of infiltrated solution used about 30 µl on sepals and petals. The experiments were repeated for 5 times. The infiltrated sepals or petals were co-cultivated for 3-5 days at 25°C and then moved to a nursery at Faculty of Agriculture, Natural Resources and Environment, Naresuan University until collection.