CHAPTER V

CONCLUSION

Orchid is an important economic flower of Thailand that the export value of orchid flowers is over 2,000 million Bahts each year. *Dendrobium* Sonia cv. Earsakul is one of popular Thai orchid in the world floriculture market as it is unique and spectacular form and color. Improvement of *D.* Sonia cv. Earsakul flower color may help increase the export value in the future. Metabolic engineering is the advanced biotechnology that can be used to alter flower color of *D.* Sonia cv. Earsakul without changing flower form via the molecular modification of anthocyanin biosynthetic pathway.

In this study, we studied the expression profiles of *DFR* in flowers of *D.* Sonia cv. Earsakul and developed transient RNAi system via agroinfiltration for determining effects of anthocyanin biosynthetic gene silencing on flower color using the *DFR*-hairpin RNA (*DFR*-hpRNA) construct as a model.

Our experiments included cloning of partial *DFR* cDNA from *D.* Sonia cv. Earsakul, determination of the expression profiles of the *DFR* gene and anthocyanin accumulation in *D.* Sonia cv. Earsakul flowers, construction of *DFR*-hpRNA binary vectors and characterization of transient *DFR*-hpRNAi silencing in flowers of *D.* Sonia cv. Earsakul.

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

We tested 4 combinations of primers, F1-R1, F1-R2, F2-R1 and F2-R2 for isolation of partial *DFR* cDNA from *D.* Sonia cv. Earsakul by RT-PCR. Approximately 485, 499, 456 and 470 bp. DNA fragments were generated from RT-PCR corresponding to the expected sizes on the nucleotide sequence of *DFR* mRNA used to design the primers. The 470-bp fragment was isolated and cloned into pGEM-TEasy vector. Nucleotide sequence analysis indicated that the 470-bp fragment is a part of *DFR* cDNA

2. Expression profiles of the *DFR* gene and anthocyanin accumulation in *D.* Sonia cv. Earsakul flowers.

We determined the expression levels of the *DFR* gene in sepals and petals of *D.* Sonia cv. Earsakul flowers at seven different developmental stages 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 expression levels of the *DFR* gene were steadily increased from flower bud stage 1 to the maximum level at flower bud stages 4 and declined after flower bud stage 5. Up-regulation of the *DFR* expression in the petals started earlier than in the sepals corresponding to the anthocyanin pigmentation which was initially observed in P1 and S2.In contrast, down-regulation of *DFR* expression in the petals occurred later than in the sepals corresponding to the pigment intensity which appeared in the petals more strongly than in the sepals of the fully-opened flower.

We also examined the expression pattern of *DFR* in purple and white tissues of the petal from flower stages 3-5. The expression level of *DFR* was very low to undetectable in the white tissues and related to the level of anthocyanin accumulation. We suggested that the purple and white tissues of the *D.* Sonia cv. Earsakul petal attributed to differential regulation of the *DFR* expression starting from early stages of the petal development.

3. Construction of *DFR*-hpRNA binary vectors.

Two *DFR*-hpRNA constructs were successfully generated using pSTARGATE and pWATERGATE vectors through gateway technology. These two *DFR*-hpRNA constructs were driven by different promoters. The ubiquitin promoter and the ARbcS promoter regulated *DFR*-hpRNA expression in pSTARGATE and pWATERGATE, respectively.

4. Characterization of transient *DFR*-hpRNAi silencing in flowers of *D.* Sonia cv. Earsakul by agroinfiltration.

We tested the efficiency of transient RNAi in flowers of *D.* Sonia cv. Earsakul with two different methods of *Agrobacterium*-mediated transient transformation, agroinjection and agroinfiltration, with *A. tumerfaciens* strain EHA105 containingp STARGATE and pWATERGATE with the *DFR*-hpRNA construct. The

results revealed that both methods could inhibit anthocyanin synthesis but transient RNAi via agroinfiltration was much more powerful than agroinjection.

Transient *DFR*-hpRNAi using either the *DFR*-hpRNA construct of pSTARGATE or the *DFR*-hpRNA construct of pWATERGATE effectively silenced the endogenous *DFR* expression and inhibited anthocyanin synthesis at the infiltrated regions of *D.* Sonia cv. Earsakul sepals and petals. The effect of transient *DFR*-hpRNAi on flower color was detected within a few days. The best flower stage suitable for transient RNAi assay was flower bud stage 2 which was just started to accumulate anthocyanins.

This transient RNAi system that we have developed is a simple and effective technique for determining effect of anthocyanin biosynthetic gene silencing on flower color of *D.* Sonia cv. Earsakul and other *Dendrobium* hybrids.