EXPRESSION PROFILES OF THE *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*) GENE IN THE SEPALS AND PETALS OF *DENDROBIUM* SONIA EARSAKUL

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Abstract

Dendrobium Sonia Earsakul is a purple-flower orchid with a white base of the sepals and petals. To improve flower color using metabolic engineering, the molecular basis of genes involved in anthocyanin biosynthesis is required. In this study, we analyzed the expression profiles of the *dihydroflavonol 4-reductase* (*DFR*) gene in the sepals and petals at different flower developmental stages. The RT-PCR analysis showed that the expression of the *DFR* gene in the sepals and petals was developmentally regulated and corresponded to anthocyanin accumulation. No transcript of *DFR* was detected in either the sepals or petals at the fully-opened flower stage. The regulation of the *DFR* expression was different between the sepals and petals at the early and late stages of flower development. In the white tissues of the petals of the flower buds, the expression of the *DFR* gene was repressed and low levels of anthocyanins were detected. This indicates that the purple and white tissues of the *D*. Sonia Earsakul petals are attributed to differential regulation of the *DFR* expression.

Keywords: Anthocyanin, *Dendrobium*, DFR, Dihydroflavonol 4-reductase, Orchid, RT-PCR

Introduction

Dendrobium Sonia Earsakul is one of the important tropical orchids of Thailand. As an exotic flower, this orchid is popular and exported to many countries around the world. *D.* Sonia Earsakul has only a purple flower with a white base of sepals and petals. Development of a new flower color with no change of the flower form is impossible by conventional breeding. Advances in plant molecular biotechnology have great potential to contribute to the breeding of a novel color flower using recombinant DNA technology or genetic engineering. The advantage of molecular breeding is that the desirable flower color can be created without interfering with other characteristics via metabolic engineering of the anthocyanin biosynthetic pathway. However, improvement of the flower color using metabolic engineering requires molecular basis of genes involved in anthocyanin biosynthesis. Flower color is mostly determined by flavonoids, which are mainly anthocyanins. The anthocyanin biosynthetic pathway has been extensively studied in many plant species. Several enzymes of this pathway have been characterized including dihydroflavonol 4-reductase (DFR), which catalyzes a key step late in the biosynthesis of anthocyanins (Figure 1). DFR is an enzyme required to produce pelargonidin-, delphinidin-, and cyanidin- based anthocyanins, which

tend to yield orange/brick red, blue/purple and red/magenta, respectively (Forkmann et al., 2001; To and Wang, 2006; Tanaka et al., 2010). *DFR* genes have been isolated and characterized in many plants (Zhang et al., 2008) including *Dendrobium* orchids (Mudalige-Jayawic karma et al., 2005; Pitakdantham et al., 2011). In some plants, such as petunia (*Petunia* hybrid) and cymbidium orchid (*Cymbidium* hybrid), DFR is substrate specific and unable to utilize dihydrokaempferol. These plants naturally lack or rarely contain pelargonidin-based anthocyanins and therefore do not produce flowers of orange/brick red colors (Meyer et al., 1987). However, suppression of *DFR* leads to the production of flowers with pale or white colors (Aida et al., 2000). In this study, we determined the expression profiles of *DFR* in the sepals and petals of *D.* Sonia Earsakul flowers at different flower developmental stages. We also demonstrated the differential expression of *DFR* between purple and white tissues of the petals and the correlation of *DFR* expression and anthocyanin accumulation.

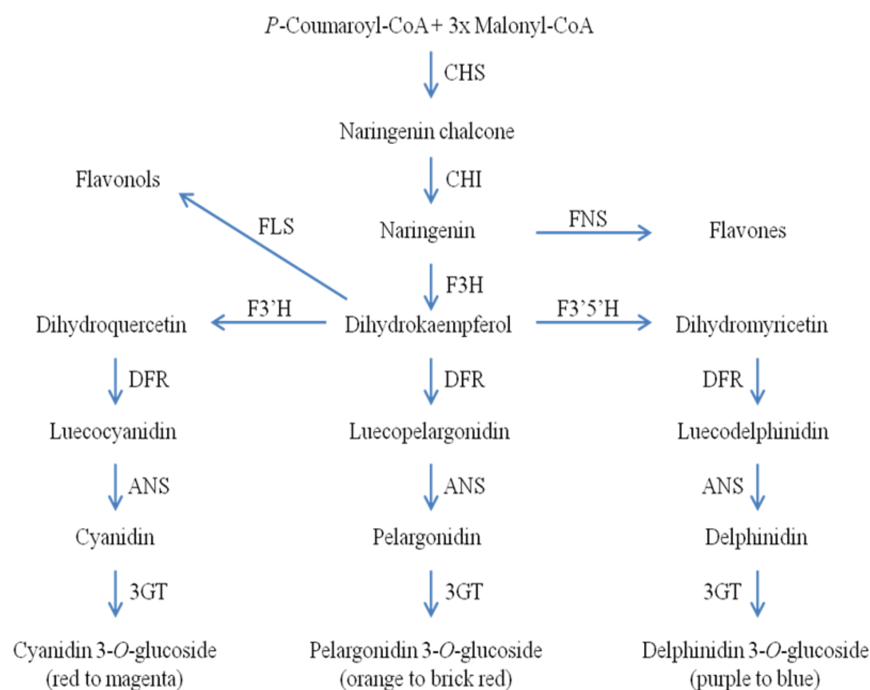


Figure 1 Schematic representation of the anthocyanin biosynthetic pathway relevant to flower color

Methodology

Plant materials

Flowering *D.* Sonia Earsakul plants were obtained from orchid farms in Nakhon Pha-tom province, Thailand. Flowers at seven different developmental stages, from buds to fully-opened flowers, were collected for RNA extraction. Developmental stages are as follows (Figure 2): Stage 1, flower bud length less than 2 cm; Stage 2, flower bud length between 2.0-2.3 cm; Stage 3, flower bud length between 2.8-3.0 cm; Stage 4, flower bud length between 3.3-3.5 cm; Stage 5, flower bud length between 3.8-4.0 cm; Stage 6, opening flower; and Stage 7, fully-opened flower.

Total RNA extraction

Total RNA extraction from the sepals and petals of *D.* Sonia 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 using a TLES buffer (0.1 M Tris-HCl, 0.1 M LiCl, 10 mM EDTA, 1% SDS, and 2% Na_2SO_3). Precipitation was carried out using 5 M LiCl at 4 °C overnight and 10% of 2.5 M CH_3COONa and 2.5 volumes of absolute ethanol at -80 °C for 60 min. 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.

RT-PCR analysis

The purified total RNA was treated with DNase prior to reverse transcription. The first strand cDNA was synthesized with the total RNA using the reverse transcription (RT) system (Promega, USA). The RT product was used as a template, with a specific forward primer, 5'-GACCCTGAGAATGAAGTG-3', and reverse primer, 5'-GAAGAGCAAATGTATCT ACC-3', which were designed based on the sequence of the *Dendrobium DFR* accession FM209431. PCR reactions were 35 cycles of 92 ºC for 30 sec, 50 ºC for 20 sec, and 72 ºC for 30 sec. To measure the relative amount of cDNA, the *Actin* transcript was used as an internal control. The primers used to amplify the *Actin* cDNA were 5'-TATTGTGCTTGATTC TGGTG-3' (forward) and 5'-AGTTGTATTGTTGTCTCGTG-3' (reverse), designed based on the sequence of the *Dendrobium Actin* accession EF612438. RT-PCR reactions were 28 cycles of 92 ºC for 30 sec, 50 ºC for 20 sec, and 72 ºC for 30 sec. PCR products were analyzed on 1.2% (w/v) agarose TAE gel electrophoresis.

Anthocyanin analysis

The anthocyanin was isolated from the flower buds of *D.* Sonia 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.

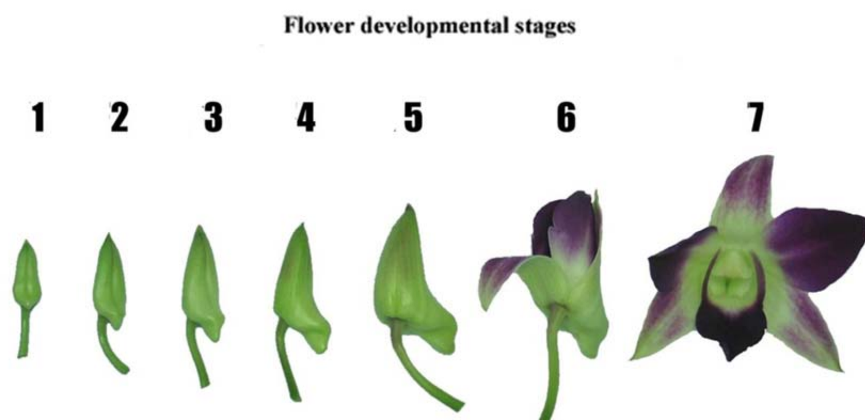


Figure 2 Flower developmental stages of Dendrobium Sonia Earsakul. Stage 1 = < 2 cm, 2 = 2.0-2.3 cm, 3 = 2.8-3.0 cm, 4 = 3.3-3.5 cm, 5 = 3.8-4.0 cm, 6 = opening flower and 7 = fully opened flower

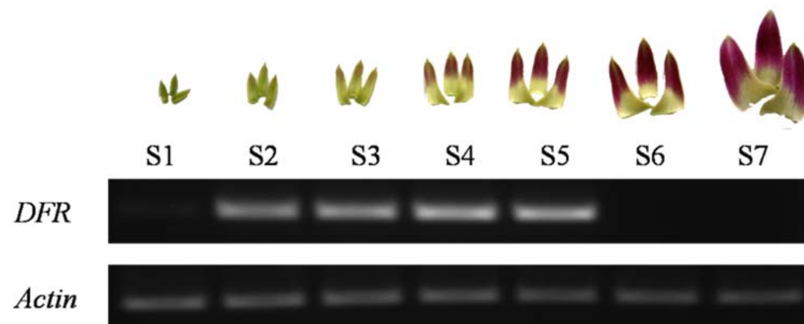
Results

Expression profiles of the *DFR* gene in *D.* Sonia Earsakul flowers

The expression levels of the *DFR* gene were investigated in the sepals and petals of *D.* Sonia Earsakul at seven different developmental stages (Figure 3). RT-PCR analysis showed that the expression patterns of *DFR* in the sepals and petals during flower development were obviously different. In the sepals, the *DFR* transcripts were detected in very small amounts at stage 1, and increased with flower development to the maximum level at stage 4. The expression levels declined to undetectable levels at stage 6 (opening) and 7 (fully opened) (Figure 3a). A different temporal expression pattern was observed in petals in that the expression level of the *DFR* transcripts was prominent at stage 1, gradually accumulating to the maximum level at stage 4, with a dramatic decrease in *DFR* transcripts observed in stage 6, and an undetectable level of *DFR* transcripts noted in the fully-opened flower stage (Figure 3b).

To investigate the tissue specificity of the *DFR* transcripts, the total RNA from the purple and white tissues of the petals was isolated separately. RT-PCR analysis was carried out at flower bud stages 3, 4 and 5. The *DFR* transcripts were not detected in the white tissues of petals from flower bud stage 3 and were detected in small amounts in the white tissues of petals from flower bud stages 4 and 5. In the purple tissues of petals, the *DFR* transcripts were detected in all stages but the levels significantly increased from stage 3 to stages 4 and 5 (Figure 4).

(a) Sepals



(b) Petals

Figure 3 RT-PCR analysis of *DFR* expression in sepals and petals of *D.* Sonia Earsakul during flower development; (a) Expression levels of *DFR* gene in sepals at seven developmental stages and (b) Expression levels of *DFR* gene in petals at seven developmental stages. *Actin* was amplified as an internal control

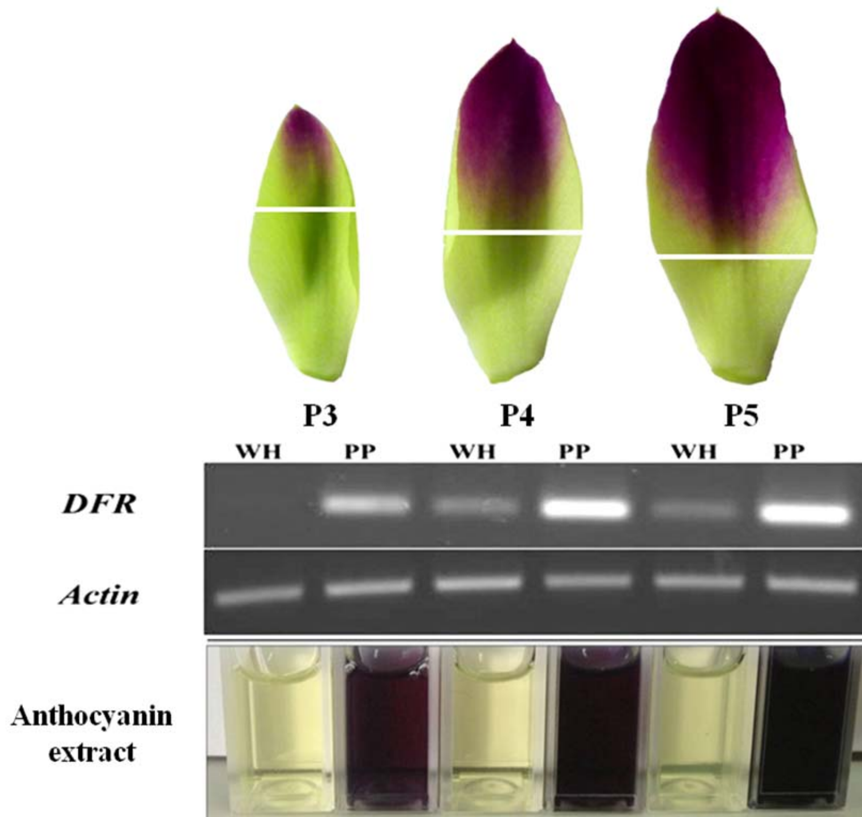


Figure 4 RT-PCR analysis of *DFR* expression and anthocyanin extracts in white and purple tissues of petals P3, P4 and P5 from flower bud stages 3, 4 and 5, respectively. *Actin* was amplified as an internal control. WH and PP indicate white and purple tissues, respectively. The white bars on petals divide white and purple tissues of each stage

Analysis of anthocyanin accumulation in the petals of *D.* Sonia Earsakul

To determine the anthocyanin accumulation in different color tissues of petals, the total anthocyanins were extracted from the purple and white tissues of petals from flower buds at stages 3, 4 and 5. In the purple tissues, anthocyanins were detected in all tested flower stages and significantly increased from stage 3 to stage 5. The anthocyanin contents were 2.5± 0.10, 6.2 ± 0.17 and 9.4± 0.56 units/g of fresh tissue from stages 3,4 and 5, respectively (Figure 5).In the white tissues, anthocyanin accumulation was very low. The anthocyanin contents were 0.066± 0.0074, 0.076 ± 0.0045 and 0.086 ± 0.0038units/g of fresh tissue from stages 3, 4 and 5, respectively (Figure 5). The accumulation levels of anthocyanins coincided with visible purple pigmentation.

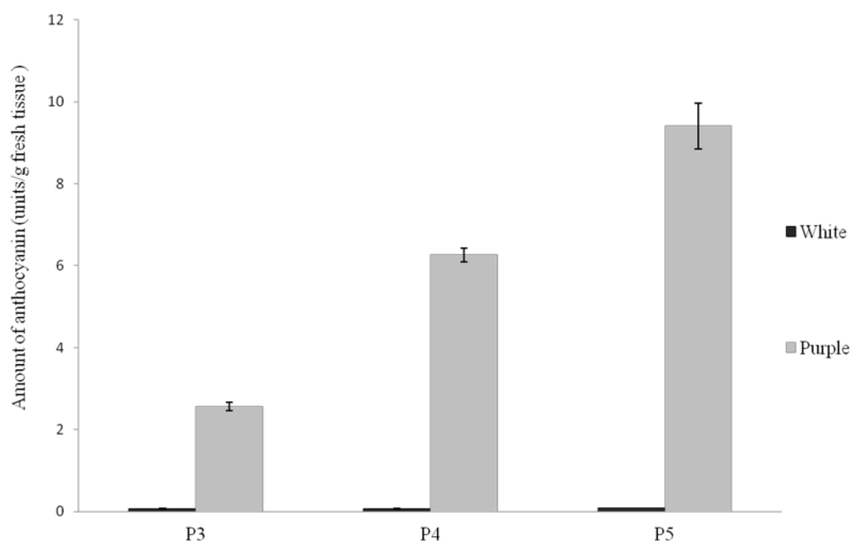
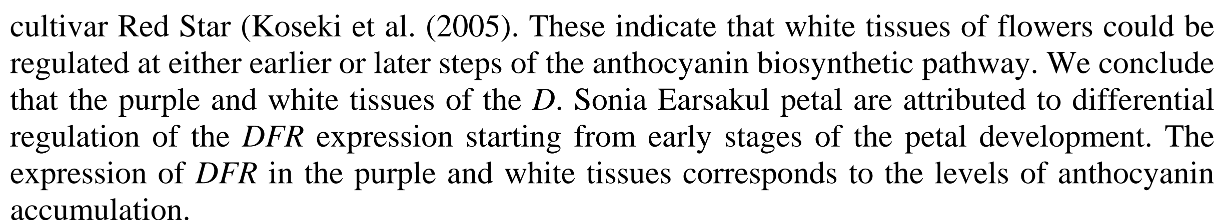


Figure 5 Quantitative analysis of anthocyanin contents in white and purple tissues of petals P3, P4 and P5 from flower bud stages 3, 4 and 5, respectively. The black bar indicates the amount of anthocyanins from white tissues and the gray bar indicates the amount of anthocyanins from purple tissues. The data represents the mean and standard errors obtained from two replicates per sample

Discussion and Conclusion

In this study, we determined the expression profiles of the *DFR* gene during the flower development of *D*. Sonia Earsakul in the sepals and petals separately. The expression of *DFR* in both sepals and petals increased and reached maximum expression in the flower bud stage before opening and declined to an undetectable level when flowers were fully opened. This indicated that the *DFR* expression in the sepals and petals of *D*. Sonia Earsakul was developmentally regulated. A similar expression pattern of the *DFR* gene has been reported in flowers of *Torenia hybrida* (Ueyama et al., 2002), *Gentiana triflora* (Nakatsuka et al., 2005), *Dendrobium* hybrid (Mudalige-Jayawickrama et al., 2005), *Petunia hybrida* (Saito et al., 2006), *Nierembergia* sp.(Ueyama et al., 2006), *Dendrobium* Sonia Earsakul (Pitakdantham et al., 2011), and *Ascocenda* spp. (Kunu et al., 2012). Our results also showed that up-regulation of the *DFR* expression in the petals of *D*. Sonia Earsakul started earlier than in the sepals. These expression patterns corresponded 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. This regulation was in accordance with the pigment intensity which appeared in the petals more strongly than in the sepals of the fully-opened flower.

Semi-quantitative analysis of the *DFR* expression levels in the white and purple tissues of the petals of *D*. Sonia Earsakul flower buds suggested that the white tissues would be the result of a block in the anthocyanin biosynthetic pathway at DFR. Ma et al. (2009) also reported that the white flower of *Phalaenopsis amabilis* had a very low expression of *DFR* whereas Liew et al. (1998) reported that *DFR* expression was detected in the red and white regions of the *Bromheadia finlaysoniana* flower. In *Petunia hybrida* cultivar Baccara Rose Picotee, the transcripts of *DFR* were detected in the white margin of the corolla at the same level as in the colored tissue whereas *CHS* transcripts were only detectable in the colored tissue (Saito et al., 2006). *CHS* repression was also found in the white sectors of the flower of *Petunia hybrid*



cultivar Red Star (Koseki et al. (2005). These indicate that white tissues of flowers could be regulated at either earlier or later steps of the anthocyanin biosynthetic pathway. We conclude that the purple and white tissues of the *D*. Sonia Earsakul petal are attributed to differential regulation of the *DFR* expression starting from early stages of the petal development. The expression of *DFR* in the purple and white tissues corresponds to the levels of anthocyanin accumulation.

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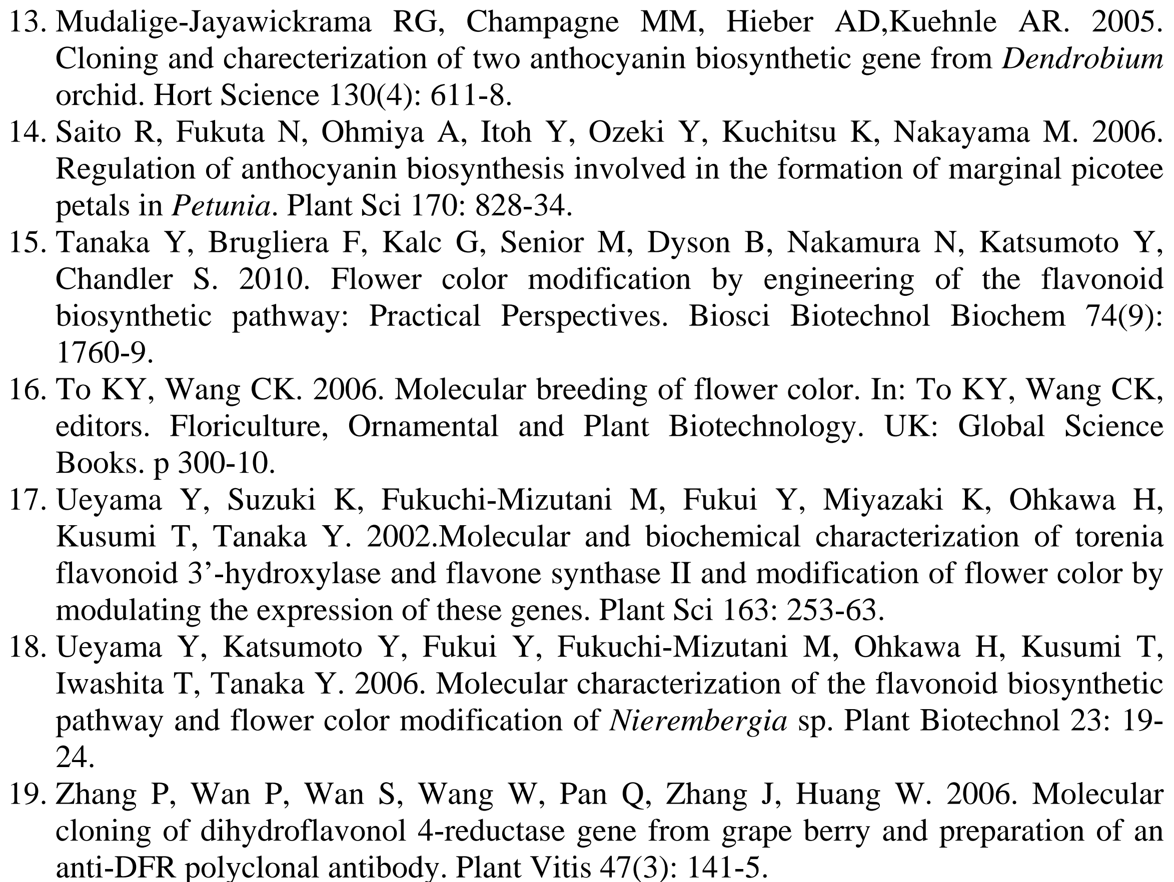

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