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Isolation and Characterization of Dihydroflavonol 4-reductase Gene in *Dendrobium* Flowers

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ABSTRACT

Dendrobium Sonia 'Earsakul' and *Dendrobium* Red Bull are widely cultivated for commercial production as cut flower orchid in Thailand. Their purple flower colors are controlled by a series of enzyme which contribute to production of anthocyanin pigments. This study was aimed to isolate dihydroflavonol 4-reductase gene, one of the key enzyme in anthocyanin synthetic pathway from two *Dendrobium* orchids and to study gene expression pattern during developmental stages of flower. A full-length cDNA clone of *DFR* gene was obtained from both *Dendrobium* cultivars. The cDNA clones contained 1,059 bp and encoded 352 amino acids. Multiple alignment of amino acid sequences showed that the dihydroflavonol 4-reductase of *Dendrobium* Sonia 'Earsakul' and *Dendrobium* Red Bull exhibited high homology to those of *Dendrobium hybrid* 'Uniwai Prince' and *Dendrobium hybrid* 'Greeting Fragrance'(99% identity). Phylogenetic tree revealed that the dihydroflavonol 4-reductase of *Dendrobium* orchids were closer relatedness than those of other orchid plants like *Bromheadia* and *Oncidium*. Relative quantification analysis of dihydroflavonol 4-reductase gene expression in floral tissues at five developmental stages of *Dendrobium* Sonia 'Earsakul' and *Dendrobium* Red Bull showed that expression of the dihydroflavonol 4-reductase gene was largely detected in young flower buds at the early stage of pigmentation. In open and fully colored flowers, transcription of the gene was decreased to an undetectable level.

Key words: Dihydroflavonol 4-reductase, anthocyanin, *Dendrobium* orchid, phylogenetic analysis, relative quantification, real time PCR

INTRODUCTION

Anthocyanin type flavonoid is a major factor in flower plant pigmentation. It contributes a wide range of colors from red to magenta, orange to red and purple (Tanaka *et al*., 2005). Analyses of anthocyanins or anthocyanidins existing in flowers and fruits were recently shown to be a benefit for selection in breeding program (Adam *et al*., 2002; Ling and Sreeramanan, 2007; Muchuweti and Chikwambi, 2008). Recently, the antioxidant activities of several bean genotypes have been

described by determination of naturally occurring variability in anthocyanin (Akond *et al*., 2011). Biosynthesis of these pigments is controlled by a series of enzymes. One of the key enzymes in anthocyanin biosynthetic pathway is dihydroflavonal 4-reductase (DFR). This enzyme reduces 4 carbonyl of colorless dihydroflavonal to give leucoanthocyanidin and subsequence to each anthocyanin pigment which are cyanidin, pelargonidin and delphinidin (Schwinn and Davies, 2004). The dihydroflavonal 4-reductase gene (*DFR*) was investigated in term of its specificity for anthocyanin product in many plant species such as in petunia, the pink color flower was enhanced from colorless flower line by transformation of *DFR* gene (Davies *et al*., 2003). In another study, a success in generated blue flower rose was achieved by down regulation the endogenous *DFR* gene and over expression of the Iris x hollandica *DFR* gene (Katsumoto *et al*., 2007).

In orchids, *DFR* gene was investigated in many commercial cultivars. Full length *DFR* gene was cloned from *Bromheadia* (Liew *et al*., 1998), *Cymbidium* `Rosannagirl Mild' (Johnson *et al*., 1999), *Oncidium* Gower Ramsey (Hieber *et al*., 2006) and *Dendrobium* (Mudalige-Jayawickrama *et al*., 2005). Transcription analysis of *DFR* gene showed that *DFR* gene was detected in purple color tissues of *Bromheadia*. In *Oncidium, DFR* expression level was up-regulated in unopened bud as well as in fully opened flowers. *Dendrobium* Sonia 'Earsakul' (*D*. Sonia 'Earsakul') and *Dendrobium* Red Bull (*D*. Red Bull) are widely cultivated for commercial production as cut flower orchid in Thailand. Their flower colors are purple and dark purple, respectively. Previously, quantification of anthocyanin pigments in pollinated flower of *D*. Sonia (Rebecca *et al*., 2008) and isolation and molecular characterization of *CHS* gene in *D*. Sonia 'Earsakul' (Pitakdantham *et al*., 2010) have been investigated. The aim of this study was to gain insight into color production of *D*. Sonia 'Earsakul' and *D*. Red Bull orchid plants. The isolation and molecular characterization of cDNA clones of *DFR* genes from flowers of the two orchid plants were carried out. BLAST analysis and phylogenetic tree were performed to find similarity of the *DFR* genes to those of orchids or other plant species. In addition, expression of *DFR* genes was investigated in floral tissues at five developmental stages.

MATERIALS AND METHODS

Plant materials: *Dendrobium* Sonia 'Earsakul' and *D*. Red Bull were cultivated in nursery at Faculty of Agriculture, Kasetsart University, Kamphang Saen Campus Thailand. The experiment was conducted at the Center of Excellence on Agricultural Biotechnology laboratory, Kasetsart University, Kamphang Saen Campus during June 2008 to January 2010.

Total RNA extraction: Floral tissues of both cultivars were utilized for total RNA extraction by modified CTAB method (Sambrook *et al*., 1989). The modification was done in nucleic acid precipitation step. The equal volume of 4 M LiCl was added to aqueous extracted solution for precipitate DNA from the extraction buffer. The 2.5 volumes of absolute ethanol and 3 M sodium acetate pH 6.0 were then used to precipitate total RNA instated of isopropanol. The total RNA was used for cDNA synthesis. For gene expression study, flower tissues of five developmental stages (Fig. 1) were separately extracted for total RNA as described above.

Cloning *DFR* genes: The first stranded cDNA was synthesized from total RNA using MMLV reverse transcriptase (Fermentas, Canada) and oligodT including M13 reverse sequence was used as a primer. The polymerase chain reaction was used to amplify the partial gene sequence using

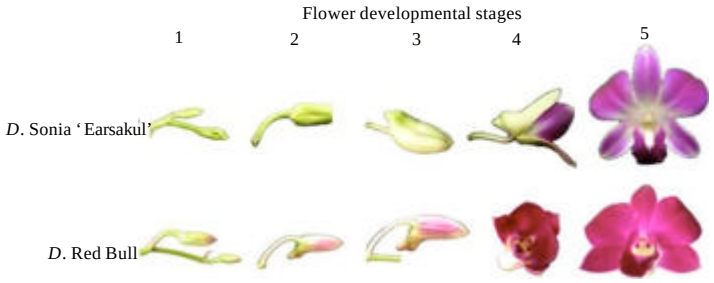

Fig. 1: Description of the flower developmental stages of *Dendrobium* orchids, 1: Small flower bud, 2: Medium flower bud, 3: Large and nearly open bud, 4: Half open flower, 5: Fully open flower

Table 1: Primers used for amplification of *DFR* and 18SrRNA genes

Primer name	Sequence 5'.... 3'	Target/Tm*
OligodT M13R	CAGGAAACAGCTATGACCATGTTTTTTTTTTTTTTT	cDNA synthesis
DFR560F	TTGCTCSAATGAACTGCTCAGCATWTGGAA	*DFR* partial gene/60°C
DFR560R	TGTGAGCATCACATAMGTCATCCAAATGAAC	
5'DFRF	GAAATGGAGAATGAGAAGAAGGGWCC	*DFR* 5' end/58°C
DFR560R	TGTGAGCATCACATAMGTCATCCAAATGAAC	
DFR633F	TATCATTAATTACAGGAAATGATGCC	*DFR* 3' end/58°C
M13R	CAGGAAACAGCTATGACCATG	
DFR101F	ATGCCACCAAGCATGATCACTGC	*DFR* quantification/58°C
DFR560R	TGTGAGCATCACATAMGTCATCCAAATGAAC	
18SrRNAF	GCTACTCGGATAACCGTAGT	*18srRNA* quantification/58°C
18SrRNAR	ACCAGACTTGCCCTCCAATG	

* Tm is an annealing temperature of each primer pair

a degenerate primer pairs. The reaction PCR mix contained 5 μL of 10 x PCR buffer, 1 μL of 50 mM $MgCl_2$, 10 μL of 1 mM dNTPs, 2 μL of 10 μM each primer (Table 1), 1 μL (5 units) of Taq polymerase (Invitrogen, USA) in a total volume of 50 μL. The reaction profile consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at an appropriate temperature for 45 sec and extending at 72°C for 1 min. After the cycles were completed, the reaction was further incubated at 72°C for 10 min. The amplified fragments were ligated to pDrive (QIAGEN, Germany) and used for the transformation of *E. coli* DH5α. All positive clones were subjected to sequencing analysis.

Sequence analysis: Nucleotide and amino acid sequences were analyzed with BLAST SEARCH (http: //blast.ncbi.nlm.nih.gov/Blast.cgi) and clustalW (http: //www.ebi.ac.uk/Tools/clustalw2/).

Phylogenetic analysis: Multiple sequence alignment was performed among DFR from various orchids and other flowering plants using clustalW program. A phylogenetic tree was constructed by the neighbor joining method of MEGA 4 (Tamura *et al*., 2007).

Quantification real time PCR analysis: The gene expression in different growth stages of flower buds was investigated by relative quantification real time PCR. The internal 18S rRNA was

used as reference control. Flower tissues of *Dendrobium* orchids at five growth stages were used for RNA extraction which was later used for real time PCR analysis. The total reaction mix of 15 μL consisted of 7.5 μL RNA direct MASTER with SYBR I dye (Invitrogen, USA), 150 ng of RNA template, 0.5 μM of each primer. The reaction profile consisted of an pre-denaturation at 95°C for 1 min, followed by reverse transcription at 61°C for 20 min and incubation at 95°C for 1 min. The amplification step consisted of 45-60 cycles of denaturing at 95°C for 20 sec, annealing at 58°C for 30 sec and extending at 72°C for 45 sec. The sections of melting were added after the cycles of amplification to obtain fluorescence measurements of non -specific products and confirm the expected DNA product by melting curve analysis. Relative changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

RESULTS

Isolation and sequence analysis of the full length *DFR* genes: In an attempt to isolate cDNA clones of *DFR* genes from flower buds of two *Dendrobium* orchids named *D.* Sonia 'Earsakul' (purple flower) and *D.* Red Bull (dark purple flower), reverse transcriptase polymerase chain reaction (RT-PCR) was performed. A partial DNA sequence of 560 bp was obtained from both *D.* Sonia 'Earsakul' and *D.* Red Bull. Blast analysis showed that the DNA fragments shared similarity to *DFR* genes of many plants including orchids. To obtain full-length clones, the 5' and 3' ends of the *DFR* genes were amplified using primers listed in Table 1. Fragments of 633 bp and 426 bp were obtained from amplification of the 3′ and 5′ends, respectively. Gene assembly revealed that a full-length cDNA clone containing an open reading frame of *DFR* gene was obtained from *D.* Sonia 'Earsakul' and *D.* Red Bull. The PCR-amplified full-length *DFR* gene fragments of *D.* Sonia 'Earsakul' and *D.* Red Bull revealed that they contain 1,059 bp encodeing 352 amino acids. They share 98% identity at both nucleotide and amino acid levels. The nucleotide sequences were deposited in Genbank database under accession number FM209431 and FM209432, respectively. Multiple alignment of amino acid sequences by ClustalW showed that DFR of *D.* Sonia 'Earsakul' and *D.* Red Bull exhibited high homology to those of *D. hybrid* 'Uniwai Prince' and *D. hybrid* 'Greeting Fragrance' (99% identity) (Fig. 2). The DFR of *D.* Sonia 'Earsakul' and *D.* Red Bull also shared homology to those of other orchid plants like *Bromheadia, Cymbidium* and *Oncidium* orchids (80-86% identity). However, they exhibited less similarity to the DFR of non-orchid plants, like petunia or gerbera (55-68% identity). A phylogenetic tree, constructed based on amino acid sequences of DFR, revealed that DFRs of *Dendrobium* orchid are clustered in the same group and DFR of orchid plants (including *Dendrobium, Bromheadia, Oncidium* and *Cymbidium* orchids) displayed closer relatedness than to those of other monocot and dicot plants.

Expression of *DFR* gene in floral tissues: Expression of *DFR* gene was examined in flowers of *D.* Sonia 'Earsakul' and *D.* Red Bull at five developing stages by qRT-PCR. Expression pattern of *DFR* gene of *D.* Sonia 'Earsakul' and *D.* Red Bull are similar (Fig. 3). Transcription of *DFR* gene in both orchid plants was not detected in small flower buds with no pigmentation (stage 1 of development). At stage 2 of flower development (medium-sized flower buds with slight pigmentation), expression of the *DFR* genes were largely detected and it was reached maximum when the buds was developed into stage 3 (nearly open flower with slight pigmentation in petals and lip). The expression was then sharply decreased when the flower bud entered to stage 4 of development (half open flowers with full pigmentation). At stage 5 in which flower was fully open and colored, expression of *DFR* gene in both orchid plants was decreased below a detectable level.

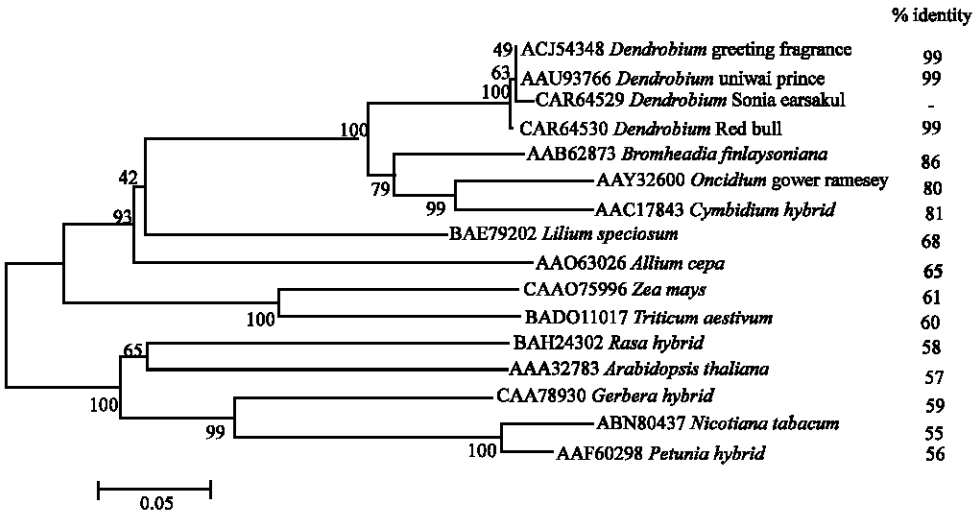

Fig. 2: The phylogenetic tree of plant DFR was generated using clustalW and MEGA4(Tamura *et al*., 2007). The identity number was showed in the right hand. Numbers next to the branches represent percentage of bootstap values from 1000 replications. The bar indicates an evolutionary distance of 0.05%. Accession numbers DFR proteins were placed in front of their names

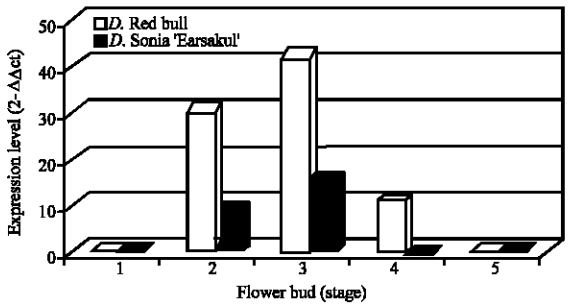

Fig. 3: Expression of *DFR* gene in flowers of two *Dendrobium* orchids at different growth stages

DISCUSSION

A cDNA sequence of *DFR* gene was isolated from *D*. Sonia 'Earsakul' and *D*. Red Bull. The nucleotide and amino acid sequences of *DFR* gene of both orchids are almost identical with 98% identity. Multiple alignments and phylogenetic tree revealed that DFR of *Dendrobium* orchids shared a high degree of homology among them and a lesser extends to other orchid plants.

Expression of *DFR* gene in *D*. Sonia 'Earsakul' and *D*. Red Bull was found in closed flower buds and it appeared to correlate with the onset of pigmentation in flower buds. Transcription of *DFR* gene was detected when flower buds started accumulation of pigment and it was highest before completion of pigmentation. In open and fully colored flowers, expression of *DFR* gene in both orchid plants was dramatically decreased to an undetectable level. The finding was consistent with the previous study in *Dendrobium* orchid of which expression of *DFR* gene was detected only in flower buds (Mudalige-Jayawickrama *et al*., 2005). In contrast, expression of *DFR* gene in *Bromheadia* and *Oncidium* was found throughout flower development (Liew *et al*., 1998;

Hieber *et al.*, 2006). In other flowering plants such as carnation and lily expression of *DFR* gene was detected in flowers at the onset of pigmentation and throughout (Hennayake *et al.*, 2007; Mato *et al.*, 2001; Nakatsuka *et al.*, 2003). A similar finding was observed in bilberry fruits (Jaakola *et al.*, 2002) where expression of DFR gene was occurred during color production steadily increased during fruit and color development.

CONCLUSION

The DFR of *D.* Sonia 'Earsakul' and *D.* Red Bull exhibited high homology and were closer relatedness to those of *Dendrobium* orchids. In this study it was shown that expression of *DFR* gene in *Dendrobium* orchid is different from other plants. Its expression was highly detected only in flower buds during the onset of pigmentation.

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