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 Fragrances’ (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 dihydroflavonol 4-reductase (DFR). This enzyme reduces 4 carbonyl of colorless dihydroflavonol to give leucoanthocyanidin and subsequent to each anthocyanin pigment which are cyanidin, pelargonidin and delphinidin (Schwinn and Davies, 2004). The dihydroflavonol 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’ (Pitakdanatham 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 MI3 reverse sequence was used as a primer. The polymerase chain reaction was used to amplify the partial gene sequence using
Flower developmental stages

1 2 3 4 5

D. Sonia ‘Earsakul’

D. Red Bull

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 18S rRNA genes

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5’…3’</th>
<th>Target/Tm*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo5T M13R</td>
<td>CAGGAAAACAGCATGACCATGTTTTTTTTTTTTTTT</td>
<td>cDNA synthesis</td>
</tr>
<tr>
<td>DFR560F</td>
<td>TTGCTCATAATGACATGCTCAGATWGGAA</td>
<td>DFR partial gene/60°C</td>
</tr>
<tr>
<td>DFR560R</td>
<td>TGTGACCATCATAMTGTCATTCGAAATGAA</td>
<td></td>
</tr>
<tr>
<td>5’DFR</td>
<td>GAAATGAGAATGAAAGAAGGWWCC</td>
<td>DFR 5’ end/58°C</td>
</tr>
<tr>
<td>DFR560R</td>
<td>TGTGAGATCATACATAMTGTCATTCGAAATGAA</td>
<td>DFR 3’ end/58°C</td>
</tr>
<tr>
<td>DFR633F</td>
<td>TATCATTTAATTACGAAATGATGCC</td>
<td></td>
</tr>
<tr>
<td>M13R</td>
<td>CAGGAAAACAGCTATGACCATG</td>
<td>DFR quantitation/58°C</td>
</tr>
<tr>
<td>DFR101F</td>
<td>ATGCCACCAACCATGATCCTGC</td>
<td></td>
</tr>
<tr>
<td>DFR560R</td>
<td>TGTGAGATCATACATAMTGTCATTCGAAATGAA</td>
<td></td>
</tr>
<tr>
<td>18S rRNA-F</td>
<td>GCTACTCGATAACCTACTG</td>
<td>18S rRNA quantitation/58°C</td>
</tr>
<tr>
<td>18S rRNA-R</td>
<td>ACCAGACTTCGCCCTCAATG</td>
<td></td>
</tr>
</tbody>
</table>

* 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 MgCl₂, 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, 0.6 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^ΔΔCt 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 425 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 encoding 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 ‘Uniwa Princess’ 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 bootstrap 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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REFERENCES


