African Journal of Biotechnology Vol. 11(64), pp. 12702-12709, 9 August, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4009
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Cloning and expression analysis of dihydroxyflavonol 4-reductase (DFR) in *Ascocenda* spp.

Wuttisak Kunu[1], Sudarat Thanonkeo[2] and Pornthap Thanonkeo[1,3]*

[1]Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand.
[2]Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham 44150, Thailand.
[3]Fermentation Research Center for Value Added Agricultural Products, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand.

Accepted 23 May, 2012

Dihydroflavonol 4-reductase (*DFR*) gene is a key gene of anthocyanins biosynthesis pathway, which represent an importance pathway for orchid flower. In this study, cloning and expression analysis of *DFR* gene in *Ascocenda* spp. were carried out. Nucleotide analysis revealed that the *Ascocenda DFR* gene was 1,056 bp in length, and encoded a protein of 351 amino acid residues. A typical translation initiation codon (ATG) and translation termination codon (TGA), the most frequently found codon in plant were identified, indicating a full-length coding sequence of the *DFR* gene. The calculated molecular mass of the deduced polypeptide was 39.8 kDa and the predicted isoelectric point was 5.58. Homology analysis revealed that the amino acid sequence of the *Ascocenda DFR* gene product was 80 to 87% identity to amino acid sequences of *DFR* gene products of other orchids such as *Bromheadia*, *Dendrobium*, *Cymbidium* and *Oncidium*. It also showed a high degree of identity to the *DFR* gene products of other flowers such as *Lilium*, *Tilipa*, *Allium*, *Gentiana* and *Chrysanthenum*. Southern blot analysis indicate that *DFR* is presented as a single copy in the *Ascocenda* spp. genome. The *AscoDFR* gene was highly expressed in the flower stages 2 and 3 of development as well as in the sepal and petal of the orchid flower.

Key words: Orchid, dihydroxyflavonol 4-reductase, anthocyanins, gene cloning.

INTRODUCTION

Anthocyanins are the primary flower pigments that belong to the most extensively studied group of plant secondary metabolites, flavonoids. They are composed of six main anthocyanidins (aglycone of anthocyanins), that is, pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Aida et al., 2000). Three different classes of anthocyanidins are responsible for the most frequent shades, that is, pelargonidin (orange to brick red), cyanidin (red to pink) and delphinidin (purple to blue) (Tanaka et al., 1998). The biosynthesis pathway for anthocyanins is well established and several enzymes involved in the biosynthesis pathway have been identified, including chalcone synthase (CHS), chalcone flavanone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), anthocyanidin reductase (ANR), and flavonoid 3-O-glucosyltransferase (UFGT). DFR (EC 1.1.1.219), which catalyzes the NADPH-dependent conversion of stereospecific dihydroflavonols such as dihydrokaempferol (DHK), dihydromyricetin (DHM) and dihydroquercetin (DHQ) into unstable corresponding leucoanthocyanidins or flavan-3, 4-diols, is a later key enzyme controlling metabolic flux leading to anthocyanins and proanthocyanidins production (Holton and Cornish, 1995; Xie et al., 2004; Zhang et al., 2008). It has been showed in previous studies that substrate specificity for the DFR enzymes varied depending on the types of anthocyanins accumulated by each plant species. In *Zea mays*, for example, DHQ is preferred for the DFR reaction (Reddy et al., 1987). The DFR from *Dianthus caryophyllus* preferentially utilizes DHM and DHQ as

*Corresponding author. E-mail: portha@kku.ac.th. Tel: +66-43-362121. Fax: +66-43-362121.

substrates, in comparison with DHK (Stich et al., 1992). In *Petunia hybrida* and *Cymbidium hybrida*, DFR is not able to efficiently convert DHK to leucopelargonidin, the precursor of pelargonidin-baesd anthocyanins (Tanaka et al., 2005). Owing to the crucial role of this enzyme in anthocyanin biosynthesis pathway, *DFR* genes have been isolated and characterized from several higher plants such as *Forsythia x intermedia* (Rosati et al., 1997), *Bromheadia finlaysoniana* (Liew et al., 1998), *C. hybrida* (Johnson et al., 1999), *Torenia fournieri* (Aida et al., 2000), *Triticum aestivum* (Himi and Noda, 2004), *Vitis vinifera* (Zhang et al., 2008), and *Brassica rapa* (Lee et al., 2008). Their regulation mechanism at transcriptional as well as translational level has also been extensively studied (Piero et al., 2006; Zhang et al., 2008).

In recent years, progress has been made in the use of genetic transformation to modify flower colour, which is one of the most important characteristics of commercially important flowers like orchid. The study of gene organization and expression profile of the *DFR* gene are essential for providing useful information for genetic transformation programs aimed at changing flower colour. An orchid species, *Ascocenda* spp., one of the most important cut flower for ornamental industrial of Thailand, was used for this purposed. Although the *DFR* gene has been cloned and characterized in several orchids such as *B. finlaysoniana* (Liew et al., 1998) and *C. hybrida* (Johnson et al., 1999), to our knowledge this is the first report on cloning, sequencing and characterization of the *DFR* gene in the *Ascocenda* flower. The expression profiles of the *DFR* gene during flower development and in different floral organs such as sepal, petal, column and labellum were also described.

MATERIALS AND METHODS

Plant materials

Orchid flowers (*Ascocenda* spp.) were collected from Bangkok Green Co., Ltd (Nakhon Pathom, Thailand). The flowers were immediately put in vase solution after harvested and then transferred to the laboratory within 6 h. Flowers at different developmental stages including stage 1, unopened buds with ca. 0.5 to 1.0 cm in length; stage 2, unopened buds with ca. 1.0 to 1.5 cm in length; stage 3, unopened bud with 1.5 to 2.0 cm in length and the flower organs slightly pigmented; stage 4, partially opened flower and stage 5, fully opened flower and different floral organs including sepal, petal, column and labellum were collected, immediately frozen in liquid nitrogen and stored at -20°C for further processing.

Total RNA extraction

Total RNA was extracted from different developmental stages of flower and floral organs by using RNeasy Plant Mini Kit (QIAGEN, Germany). The extraction of total RNA was carried out essentially as recommended by the manufacturer's instruction except that RNase-free DNase I was added to a final concentration of 0.1 mg/ml to remove any contamination genomic DNA.

Gene amplification, cloning and characterization

Amplification of the *Ascocenda DFR* gene was performed by Reverse Transcription-PCR (RT-PCR) using degenerated oligonucleotide primers designed based on the *DFR* sequences of the *Dendrobium* hybrid cultivar Ear sa kul (FM209431), *Dendrobium* hybrid cultivar Red bull (FM209432), *Dendrobium* hybrid cultivar Geeting Fragrance (FJ426271). An internal fragment of the *DFR* gene was amplified using the forward AsDFR-F1 (5'-GCAGGAACAGTTAACGTGGA-3') and reverse AsDFR-R1 (5'-TTCTTCTTTCACTTAAC(A/T)GC-3'). The RT-PCR reaction was carried out using OneStep RT-PCR Kit (QIAGEN, Germany). The reaction mixture (50 µl) consisted of 10 µl of 5x QIAGEN OneStep RT-PCR buffer, 400 µM dNTP, 1 µl of each oligonucleotide primer (forward and reverse primers), 2 µl of QIAGEN OneStep RT-PCR Enzyme mix and 1 µg of RNA template. The RT reaction was carried out at 50°C for 30 min using the Icycler Thermal Cycler (BIO-RAD, USA). Following an initiation denaturation of template cDNA at 95°C for 15 min, 40 cycles of the following temperatures were used: denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. The PCR product was separated on 0.7% agarose gel, then target DNA was excised from the gel and purified using the NucleoSpin Extract II Kit (Macherey-Nagel, Germany). The purified PCR product was cloned into pGEM T-easy vector (Promega, Madison, WI, USA) and transformed into the competent *Escherichia coli* JM109 using the Rapid DNA Ligation and Transformation Kit (Fermentas, USA). After screening, target DNA was sequenced by the dideoxynucleotide chain termination method using the MegaBACE 1000 automated DNA sequencer (Pharmacia Biotech, Sweden). In order to obtain the sequence of 5'- and 3'-end of the *DFR* gene, the SMART^{TM} RACE cDNA Amplification Kit (ClonTech, USA) was used. The reaction was carried out essentially as recommended by the manufacturer's instruction. The complete full length open reading frame (ORF) of the *DFR* gene was confirmed by RT-PCR using the forward AsDFR-F2 (5'-ATGGAGAATGAGAAGAAGGG-3') and reverse AsDFR-R2 (5'- TTCTTCTTTCACTTAACTGC-3'). After amplification reaction, the amplified product was separated on the gel, purified, cloned and sequenced as previously described. The sequence of the *DFR* gene and deduced amino acid sequence was analyzed using GENETYX (Software Development, Tokyo, Japan), while homology searching was performed using FASTA and BLAST program in the GenBank and DDBJ databases.

Southern blot analysis of *Dihydroflavonol 4-reductase (DFR)* gene in *Ascocenda* spp.

Southern blot analysis was performed to estimate the number of genes encoding for DFR in *Ascocenda* spp. Genomic DNA (20 µg) isolated from petals was digested with *Pst*I, *Xba*I, *Eco*RI and *Bam*HI, electrophoresed on 0.7% agarose gel and transferred to Hybond N^+ positively charged nylon membrane (Schleicher & Schuell) by overnight capillary transfer. The DNA was cross-linked to the membrane by UV irradiation. All procedures were performed according to the standard methods as described by Sambrook and Russell (2001). To prepare a probe for Southern hybridization analysis, an internal fragment encoding the amino acid sequence of the *DFR* gene product was amplified by RT-PCR using total RNA isolated from *Ascocenda* spp. as a template and forward AsDFR-F1 and reverse AsDFR-R1 primers. The *DFR* PCR fragment was alkaline phosphatase labeled using Gene Images AlkPhos Direct Labeling and Detection System (AlkPhos DirectTM, Amersham). All procedures for DNA labeling were carried out as recommended by the manufacturer.

The nylon membrane with cross-linked DNA was prehybridized at 50°C for 2 h in hybridization oven (Hybrid limited equipment class I)

with gentle rotation. After incubation, the labeled-DNA probe was added and subsequently incubated at 55°C an overnight. The membrane was washed according to the standard procedure for Southern blot analysis. After washing, the CDP-*Star* detection reagent (AlkPhos Direct™, Amersham) was added onto the membrane and left at room temperature for 5 min. The membrane was then exposed to X-ray film (Hyper film, Amersham) for 1 h, thereafter it was developed and fixed with developer and fixer solution (Kodak), respectively.

Expression analysis of *dihydroflavonol 4-reductase (DFR*) gene by reverse transcription polymerase chain reaction (RT-PCR)

The expression level of the *Ascocenda DFR* gene was determined by RT-PCR. Total RNA was isolated from petals of orchid flowers at different developmental stages (stage 1 – 5) and from different floral organs including sepal, petal, column and labellum. RT-PCR was carried out using OneStep RT-PCR Kit (QIAGEN, Germany) with the forward AsDFR-F2 and reverse AsDFR-R2 primers synthesized based on the 5'- and 3'-region of the *Ascocenda DFR* gene. The reaction mixture (50 µl) consisted of 10 µl of 5x QIAGEN OneStep RT-PCR buffer, 400 µM dNTP, 1 µl of each oligonucleotide primer (forward and reverse primers), 2 µl of QIAGEN OneStep RT-PCR Enzyme mix and 1 µg of RNA template. As a control, 10 µg samples of total RNA were subjected to agarose gel electrophoresis (0.9% agarose) and stained with ethidium bromide. Actin gene was used as an internal control. A thermocycler was used to perform 1 cycle of 30 min at 50°C for reverse transcription following by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. The amplified products at the 20th, 23rd, 26th, 29th, 32nd and 35th cycle were electrophoresed on a 0.9% agarose gel. After staining with ethidium bromide, the relative amounts of the products were compared using the Gel Image Master (Pharmacia Biotech). The experiment was repeated at least twice. Under these conditions, the OneStep RT-PCR was able to specifically detect mRNA because no band was observed when reverse transcriptase was omitted.

RESULTS AND DISCUSSION

Cloning and sequencing analysis of the *dihydroflavonol 4-reductase (DFR)* gene from *Ascocenda* spp.

The *DFR* gene of the *Ascocenda* spp. was cloned as described in materials and methods. After sequencing, a complete full length open reading frame encoding the entire amino acid sequence of the *DFR* gene product was obtained. The nucleotide sequence of this open reading frame, designated as *AscoDFR*, its flanking region and the deduced amino acid residues are shown in Figure 1. The *AscoDFR* contained 1,056 bp in length, and encoded a protein of 351 amino acid residues. The calculated molecular mass of the deduced polypeptide was 39.77 kDa and the predicted isoelectric point was 5.58. A typical translation initiation codon (ATG) and translation termination codon (TGA), the most frequently found codon in plant, were identified, indicating a full-length coding sequence of the *DFR* gene. A putative polyadenylation signal AATCAA was found 113 nucleotides downstream of the stop codon (Joshi, 1987). The highly conserved regions among all *DFR* sequences were identified in the *AscoDFR*. One of the conserved regions at the N-terminal of the *Ascocenda* DFR sequence contained a putative NADP binding region (Lacombe et al., 1997), like that reported in other orchids. The binding site of DHQ, DHM and DHK (Johnson et al., 2001) was also found in the *AscoDFR* gene product. The conserved amino acid asparagine (N^{135}) essential for DFR activity was found in the active site of DFR sequence, like DFR from *Z. mays* and *Antirrhinum majus* (Farzad et al., 2003). DFR from *Viola cornuta* and *P. hybrida* has been showed to have no activity on DHK since the conserved amino acid within its active site was replaced with glutamic acid residue (Johnson et al., 2001; Farzad et al., 2003). Based on these findings, we speculate that the *Ascocenda* DFR can utilize DHK, a part from DHQ and DHM, and convert it into leucopelargonidin, the precursor of pelargonidin-based anthocyanins which provides the basis for orange-red hues. The exact molecular mechanism for substrate specificity of DFR has not yet been carried out in *Ascocenda* flower, therefore further research such as site direct mutagenesis or structural analysis of DFR is needed to clarify this speculation.

The deduced amino acid sequence of the *Ascocenda* DFR showed high amino acid identity to the DFR from other orchids such as *B. finlaysoniana* (AF007096) (87% identity), *D.* hybrid cultivar Ear sa kul (FM209431) (80% identity), *C. hybrida* (AF017451) (80% identity) and *Oncidium* Gower Ramsey (AY953937) (80% identity) (Figure 2). Three amino acid changes (V/I, Q/R/K and K/E) at the putative NADP binding region were observed between the species, suggesting that the DFR(s) from these orchids are different which might be due to a difference in the plant genotype. A comparison of the DFR from other plant species in the GenBank database with AscoDFR showed that AscoDFR had 68% amino acid sequence homology to DFR from *Lilium* hybrid cultivar Star Gazer (AB058641) and *Tulipa gesneriana* (AB456682), 64% to DFR from *Allium cepa* (AY221249), 63% to DFR from *Gentiana triflora* (D85185) and 62% to DFR from *Chrysanthemum x morifolium* (EF094935) (Figure 3). The amino acid sequences for putative NADP binding region of DFR from these plant species were found. However, eight amino acid changes (V/A, S/G, Y/F, V/I, V/A, K/R, Q/E/R/H and Y/N/E/R) were observed between the species.

Southern analysis of *AscoDFR* gene

Southern blot analysis was carried out to evaluate the copy number of *DFR* gene in the genome of *Ascocenda* spp. Genomic DNA isolated from petals was digested separately with *Pst*I, *Xba*I, *Eco*RI and *Bam*HI, and probed with an internal fragment of *AscoDFR*. A single

```
ATGGAGAATGAGAAGAAGGGTCCGGTTGTGGTGACGGGAGCCAGTGGGTACATCGGTTCT      60
M  E  N  E  K  K  G  P  V  V  V  T  G  A  S  G  Y  I  G  S
TGGCTAGTCATGAAGCTTCTTCGGGAGGGTTATGAGGTCAGGGCTACAGTCAGAGATCCA     120
W  L  V  M  K  L  L  R  E  G  Y  E  V  R  A  T  V  R  D  P
TCAAGTCCTAAAAAAGTGAAGCCGTTATTGGATCTCCCACGCTCGAACGAACTGCTCAGC     180
S  S  P  K  K  V  K  P  L  L  D  L  P  R  S  N  E  L  L  S
ATATGGAAAGCAGATCTAAATGACATTGAAGGGAGCTTCGATGAGGTGATACGTGGCTGT     240
I  W  K  A  D  L  N  D  I  E  G  S  F  D  E  V  I  R  G  C
GTTGGGGTGTTCCATGTCGCTACTCCCATGAATTTTCAATCCAAAGACCCCGAGAAGGAA     300
V  G  V  F  H  V  A  T  P  M  N  F  Q  S  K  D  P  E  K  E
GTGATAGAACCGGCAATCAAGGGTTTGCTGAGCATCCTGAGGTCATGCAAAAGGTCAGGC     360
V  I  E  P  A  I  K  G  L  L  S  I  L  R  S  C  K  R  S  G
AGCGTAAAGCGCGTGATATTCACATCTTCCGCAGGAACAGTTAACGTGGAGGAACACCAA     420
S  V  K  R  V  I  F  T  S  S  A  G  T  V  N  V  E  E  H  Q
GCACCGGTCTACGACGAGACCTCCTGGAGCGACCTCGATTTCATCACCAGAGTCAAGATG     480
A  P  V  Y  D  E  T  S  W  S  D  L  D  F  I  T  R  V  K  M
AACGGTTGGATGTACTTCGTATCAAAAACACTTGCGGAGAAGGCTGCTTGGGAGTTTGTG     540
N  G  W  M  Y  F  V  S  K  T  L  A  E  K  A  A  W  E  F  V
AGAGAAAATGAGATTGATTTTATAGCCATAATTCCCACTTTGGTGGTGGGTTCCTTCATA     600
R  E  N  E  I  D  F  I  A  I  I  P  T  L  V  V  G  S  F  I
ACAAATGAAATGCCACCAAGTTTGATCACTGCATTTTCATTAATTACAGGAAATGAAGCC     660
T  N  E  M  P  P  S  L  I  T  A  F  S  L  I  T  G  N  E  A
CATTACTCGATAATAAAGCAAGGTCAATTTGTTCATTTGGATGACTTATGTGATGCTCAC     720
H  Y  S  I  I  K  Q  G  Q  F  V  H  L  D  D  L  C  D  A  H
ATTTTCCTTTTCGAGCATCCCGAAGCAAATGGTAGGTACATTTGTTCTTCACATGATTCA     780
I  F  L  F  E  H  P  E  A  N  G  R  Y  I  C  S  S  H  D  S
ACAATTTATGACTTGGCCAAAATGCTGAAGAAGAGATATGCCACATATGCCATTCCTCAA     840
T  I  Y  D  L  A  K  M  L  K  K  R  Y  A  T  Y  A  I  P  Q
AAGTTTAAGGACATTGATCCAAATATTAAGAGTGTAAGTTTCTCTTCTAAGAAGTTAATG     900
K  F  K  D  I  D  P  N  I  K  S  V  S  F  S  S  K  K  L  M
GACTTGGGGTTCAAGTACAAGTACACTATTGAGGAGATGTTTGATGATGCTATTAAGACC     960
D  L  G  F  K  Y  K  Y  T  I  E  E  M  F  D  D  A  I  K  T
TGTAGGGAAAAGAATCTCTTACCACCCAACACTGAAGAACCAACCTTAGTTGAAGAGAAA    1020
C  R  E  K  N  L  L  P  P  N  T  E  E  P  T  L  V  E  E  K
TATGAAGAAGGTGAAAGAACAATTGCAGTTAAGTGAAAGAAGAATAAGCAGCTTGTAAAT    1080
Y  E  E  G  E  R  T  I  A  V  K  *
TCTTTCTGTTATCCTTTTATTTACACATGTACCTTATTATGCATGGTTATATCTTGACTG    1140
ATTATAGTGGTCTTTATTTTGATTTATTTAATGAATCATGTAATTACACACATAATCAAT    1200
TGTAATTCATGATTGTAATTTTGAATTTATATTTTGAGCTATTTAGTGGAAAAAAAAAAA    1260
AAGTACTAGTCGACGCGTGGCC                                          1282
```

Figure 1. Nucleotide and deduced amino acid sequences of the *Ascocenda DFR* gene. Nucleotides are numbered from the first nucleotide from 5' end of the sequence. Amino acids are indicated below the nucleotide sequence in single-letter codes. Translation stop codon is indicated by an asterisk. Putative polyadenylation signal is double underlined and putative NADP binding region is shaded. The proposed binding site of DHQ, DHM and DHK is underlined and the conserved amino acid asparagines and glutamine in all DFR sequences are indicated in the boxes.

hybridization band was observed in each digestion, suggesting that the *AscoDFR* gene is present as a single copy in the genome of *Ascocenda* spp. (Figure 4). This result is in agreement with that of *B. finlaysoniana* (Liew et al., 1998) and *C. hybrida* (Johnson et al., 1999), in which a single copy of the *DFR* gene has been reported. This finding raises the possibility that anthocyanin production in orchids is dependent on a single *DFR*. Therefore, biochemical properties of this single DFR protein can potentially affect the entire range of anthocyanins produced, as reported by Johnson et al. (1999).

Expression analysis of the *AscoDFR* gene

It has been reported in several plant species that the expression of *DFR* gene occurs during different developmental stages of flowers. Therefore, the *AscoDFR* gene expression in *Ascocenda* spp. was compared during

```
Bromheadia      MENEKKGPVVVTGASGYVGSWLVMKLLQKGYDVRATIRDPTNLEKVKPLL 50
Ascocenda       MENEKKGPVVVTGASGYIGSWLVMKLLREGYEVRATVRDPSSPKKVKPLL 50
Dendrobium      MENEKKGPVVVTGASGYVGSWLVMKLLKKGYVVRATVRDPTNLKKVKPLL 50
Cymbidium       METERKGPVVVTGASGYVGSWLVMKLLQKGYEVRAAVRDSTNFEKVKPLL 50
Oncidium        MGIENKGTVAVTGASGYVGSWLVMKLLQKGYEVRATVRDPTNFEKVKPLL 50
                *  *.**.*.*******:*********::** ***::**.:. :******

Bromheadia      DLPRSNELLSIWKADLNDIEGSFDEVIRGCVGVFHVATPMNFQSKDPENE 100
Ascocenda       DLPRSNELLSIWKADLNDIEGSFDEVIRGCVGVFHVATPMNFQSKDPEKE 100
Dendrobium      DLPRSNELLSIWKADLDDVEGSFDEVIRGSIGVFHVATPMNFQSKDPENE 100
Cymbidium       DLPGSNELLSIWKADLNDIDETFDEVTRGSVGLFHVATPMNFQSEDPENE 100
Oncidium        DLKGSNELLSIWKADLNDINESFDDVTRGCVGIFHVATPMNFQSKDPENE 100
                **  ************:*:: :**:* **.:*:***********:***:*

Bromheadia      VIKPAINGLLGILTSCKKAGSVKRVIFTSSAGTVNVEEHQAAVYDENSWS 150
Ascocenda       VIEPAIKGLLSILRSCKRSGSVKRVIFTSSAGTVNVEEHQAPVYDETSWS 150
Dendrobium      VIKPAINGLLGILRSCKKAGSVQRVIFTSSAGTVNVEEHQAAVYDESCWS 150
Cymbidium       VIKPTISGLLGILRSCKRVGTVKRVIFTSSAGTVNVEEHQATVYDESSWS 150
Oncidium        VIKPAINGMLGILRSCKRAGTVKRVIFTSSAGTVNVEEHQAEVYDESSWS 150
                **:*:*.*:*.** ***: *:*:***************** ****..**

Bromheadia      DLHFVTRVKMTGWMYFVSKTLAEKAAWEFVKENAIHFIAIIPTLVVGSFI 200
Ascocenda       DLDFITRVKMNGWMYFVSKTLAEKAAWEFVRENEIDFIAIIPTLVVGSFI 200
Dendrobium      DLDFVNRVKMTGWMYFLSKTLAEKAAWEFVKDNDIHLITIIPTLVVGSFI 200
Cymbidium       DLDFVTRVKMTGWMYFVSKTLAEKAAWEFVSDNDIHFITIIPTLVVGSFL 200
Oncidium        DLEYITRVKMTGWMYFLSKTLAEKAAWEFVRDNDIHFITIIPTLVMGSFL 200
                **.::.****.*****:************** :* *.:*:******:***:

Bromheadia      TNEMPPSLITALSLISGNEAHYSILKQAQFVHLDDLCDAHIFVYEHPEAN 250
Ascocenda       TNEMPPSLITAFSLITGNEAHYSIIKQGQFVHLDDLCDAHIFLFEHPEAN 250
Dendrobium      TSEMPPSMITALSLITGNDAHYSILKRVQFVHLDDVCDAHIFLFEHPKAN 250
Cymbidium       ISRMPPSLITALSLITGNEAHYSILRQAQFVHLDDLCDAHIFLFEHHKAN 250
Oncidium        ISGMPPSMITALSLITGNEAHYSIIKQAQFVHLDDLCDAHIFLFEHPEAN 250
                . ****:***:***:**:*****::: *******:******::** :**

Bromheadia      GRYICSSHDSTIYDLANMLKNRYATYAIPQKFKEIDPNIKSVSFSSKKLM 300
Ascocenda       GRYICSSHDSTIYDLAKMLKKRYATYAIPQKFKDIDPNIKSVSFSSKKLM 300
Dendrobium      GRYICSSYDSTVYGLAEMLKNRYPTYVIPQKFKEIDPDIKCVSFSSKKLL 300
Cymbidium       GRYICSSHDSTIYSLAKMLKNRYATYDIPLKFKEIDPNIESVSFSSKKLL 300
Oncidium        GRYICSSHDSTIYGLAKKLKNRYVTYAIPQKFKDIDPDIKSVSFSSKKLM 300
                *******:***:*.**: **:** ** ** ***:***:*:.********:

Bromheadia      DLGFKYKY--TIEEMFDDAIKTCRDKNLMPLNT-EELVLAAEKYDEVKEQ 347
Ascocenda       DLGFKYKY--TIEEMFDDAIKTCREKNLLPPNT-EEPTLVEEKYEEGERT 347
Dendrobium      ELGFKYKY--SMEEMFDDAINTCRDKKLIPLNTDQEIVLAAEKFEEVKEQ 348
Cymbidium       DLGFKYKYKYTMEEMFDDAIKTCRDKNLIPLHT-EEMVSANEKFDEVKEQ 349
Oncidium        DLGFKYKY--TMEEMFDDAIKACRDKNLIPLNT-EKMVSAAEKFNEIKEK 347
                :*******  ::*********::**:*:*:* :* :: . . **::* :.

Bromheadia      IAVK--- 351
Ascocenda       IAVK--- 351
Dendrobium      IAVK--- 352
Cymbidium       IAVK--- 353
Oncidium        FCLVNNE 354
                :.:
```

Figure 2. Comparison of the deduced amino acid sequence of the DFR from *Ascocenda* spp. with DFR from *Bromheadia finlaysoniana* (AF007096), *Dendrobium* hybrid cultivar Ear sa kul (FM209431), *Cymbidium hybrida* (AF017451), and *Oncidium* Gower Ramsey (AY953937). Amino acid residues identical and similar to each other are illustrated by asterisks and dots, respectively. Gaps introduced for alignment are indicated by a horizontal dash. The putative NADP binding region is shaded.

five developmental stages from unopened bud to fully opened flower. As shown in Figure 5, the accumulation level of the *AscoDFR* transcripts occurred at early stage (unopened bud) and reached its maximum at flowers stage 2 (unopened bud with ca. 1.0 to 1.5 cm in length) and stage 3 (unopened bud with 1.5 to 2.0 cm in length and the flower organs slightly pigment), where pigment development clearly occurred. Following this stage, the accumulation level of the *AscoDFR* transcripts was gradually decreased, suggesting that this gene was

```
Lilium            -MEN----VKGPVVVTGASGYVGSWLVMKLLQYGYTVRATVRDPRDLRKTKPLLDLPGAD 55
Tulipa            -MKV----VKGPVVATGASGYVGSWLVMKLLENGYTVRATVRDPKDQGKTKPLLDLRGAD 55
Ascocenda         -MENE---KKGPVVVTGASGYIGSWLVMKLLREGYEVRATVRDPSSPKKVKPLLDLPRSN 56
Allium            MMKEIG-AAGGAVVVTGAGGYVGSWLVMKLLHYGYTVRATLRDSSDEAKTKPLLELPGAD 59
Gentiana          -MEGGILSNATTVCVTGASGYIGSWLAMRLLERGYTVRATVRDPGNLKKVQHLLELPKAS 59
Chrysanthemum     -MKE---DSPAIVCVTGASGFIGSWLVMRLLERGYIVRATVRDPGDMKKVKHLLELPKAE 56
                   *:         * .***.*::****.*:**. ** ****:**. .  *.: **:*  :.

Lilium            ERLTIWKADLSE-DGSFDEAINGCTGVFHVATPMDFDSEDPENEVIKPTINGVIGIMKSC 114
Tulipa            ERLTIWKADLND-EGSFDNAINGCTGVFHVATPMDFESKDPENEVIKPTVNGVLSIMKSC 114
Ascocenda         ELLSIWKADLNDIEGSFDEVIRGCVGVFHVATPMNFQSKDPEKEVIEPAIKGLLSILRSC 116
Allium            TRLSLWEADLLQ-DGSFDHVISGSIAVFHVATPMDFDSIDPENEVIKPAVNGMLSIMKSC 118
Gentiana          TNLTLLKADLTE-EGSFDEAIHGCHGVFHVATPMDFESKDPKNEVIKPTIDGFLSIIRSC 118
Chrysanthemum     TNLTLWKADLAL-EGSFDEAIEGCHGVFHVATPMDFESKDPENEIIKPTIEGVLSIIRSC 115
                    *:: :***   :****..* *. .********:*:* **::*:*:*::.*.:.*::**

Lilium            KKAGTVKRIIFTSSAGTVNVHEHQMPEYDESSWSDIDFIRRVKMTGWMYFVSKILAEKAA 174
Tulipa            KKAGTVKRVIFTSSAGTVNVQEEQMPEYDEDSWSDIDFCRRVKMTGWMYFVSKTLAEKAA 174
Ascocenda         KRSGSVKRVIFTSSAGTVNVEEHQAPVYDETSWSDLDFITRVKMNGWMYFVSKTLAEKAA 176
Allium            KKAGTVKRVIFTSSAGTVNVEEHQKPEYDENSWSDIDFCRRVKMTGWMYFVSKSLAEKAA 178
Gentiana          VKAKTVKKLVFTSSAGTVDVQEQQKPVYDENDWSDLDFINSTKMTGWMYFVSKILAEKAA 178
Chrysanthenum     VKAKTVKKLVFTSSAGTVNVQKQQVPVYDESHWSDLDFIYSKKMTAWMYFVSETLAEKAA 175
                   :: :**:::********:*.:.* * ***  ***:**    **..******: ******

Lilium            WDFAKENDIQFISIIPTLVVGPFITTTMPPSMLTALSLITGNEAHYSILKQIQLVHLDDV 234
Tulipa            WEFAKDNDIQLISIIPTLVVGPFITTSMPPSMITALSLITGNDSHYSILKQIQLVHLDDL 234
Ascocenda         WEFVRENEIDFIAIIPTLVVGSFITNEMPPSLITAFSLITGNEAHYSIIKQGQFVHLDDL 236
Allium            WEFAKANGIDLVTIIPTLVVGAFITTAMPPSMITALSLITGNEAHYSIIKQAQLVHLDDL 238
Gentiana          WEVTKANDIGFISIIPTLVVGPFITTTFPPSLITALSLITGNEAHYGIIKQGQFVHLDDL 238
Chrysanthemum     WKATKENNIDFISIIPTLVVGPFISPSFPPSLMTALSLITGAESHYSIIKQCQCVHLDDL 235
                  *. .: * * :::********.**:  :***::**:***** ::**.*:** * *****:

Lilium            CNAHIFLFENPEAIGRYICSSYDTTIYDLARKIKDRYPQYAIPQKFEGIDDQIKPVHFSS 294
Tulipa            CIAHIFLFENQEASGRYICSSFDATIWDLARLMKDRYPQYAIPQEFEDIDEKIKPVRFSS 294
Ascocenda         CDAHIFLFEHPEANGRYICSSHDSTIYDLAKMLKKRYATYAIPQKFKDIDPNIKSVSFSS 296
Allium            CEAHILLLNHPKAEGRYICSSHDVTIYDMAKMIRQNYPQYYIPQQFEGIDKGIQPVRFSS 298
Gentiana          CEAHIFLYEHPEAEGRYICSSHDTTIHDLAKMIRQNWPEYYIPTKLKGIDEDIPVVSFSS 298
Chrysanthemum     CESHIYLYENPKAAGRYICSSHDATIHQLAKMIKEKWPEYQVPAKFEGIDDEIPIVSFSS 295
                  * :** * :: :* *******.* ** ::*: ::..:. * :* :::.**  *  * ***

Lilium            KKLIDLGFKYQYTFEEMFDEGIRSCIEKNLIPRQTQERYYADDKLNL------------- 341
Tulipa            KKLMDLGFNYQYTIEEMFDEGIRSCTEKKLLPIHTQELFYIDDKIDLGGRKKNLINEMMR 354
Ascocenda         KKLMDLGFKYKYTIEEMFDDAIKTCREKNLLPPNTEEPTLVEEKYEE------------- 343
Allium            KKLVDLGFRYKYSMESMFDEAIKTCVERKFIPLQTAVELQLKPYELLEHNNKNG------ 352
Gentiana          NKLIDLGFQYKYTLEDMFRGAIDTCKEKRMLPLSIGHQKE----STDPEVD--------- 345
Chrysanthemum     KKLTDMGFKFKYDLEEMFRGAIKSCKEKGLLPYSTNETKEGLVSSLVTKAD--------- 346
                  :** *:**.::* :*.**  .* :* *: ::*

Lilium            --------------------CCTK------------MTNDKLDLGGMKLNSLDEIVRGHN 369
Tulipa            GQSEQLSMYTVEKMFGEQIRSCTESKLLPLQTQELFYINDKIDLGGSKMNSIKEMMRGQT 414
Ascocenda         ----------------------------------------------GERTIAVK------- 351
Allium            ------------------------------------VVTNTIKIVGQMVN-TKAMITEHE 375
Gentiana          ---------------------------------------------EVVKEMELIQDSLD---- 359
Chrysanthemum     ---------------------------------------------THEEEKLQIIHKVATHPL 364
                                                                             .

Lilium            EQVSVALQ-- 377
Tulipa            EQLSTAFH-- 422
Ascocenda         ---------- 
Allium            ENEPIATH-- 383
Gentiana          ---------- 
Chrysanthemum     APLAKSILGE 374
```

Figure 3. Comparison of the deduced amino acid sequence of the DFR from *Ascocenda* spp. with DFR from *Lilium* hybrid cultivar Star Gazer (AB058641), *Tulipa gesneriana* (AB456682), *Allium cepa* (AY221249), *Gentiana triflora* (D85185) and *Chrysanthemum x morifolium* (EF094935). Amino acid residues identical and similar to each other are illustrated by asterisks and dots, respectively. Gaps introduced for alignment are indicated by a horizontal dash. The putative NADP binding region is shaded.

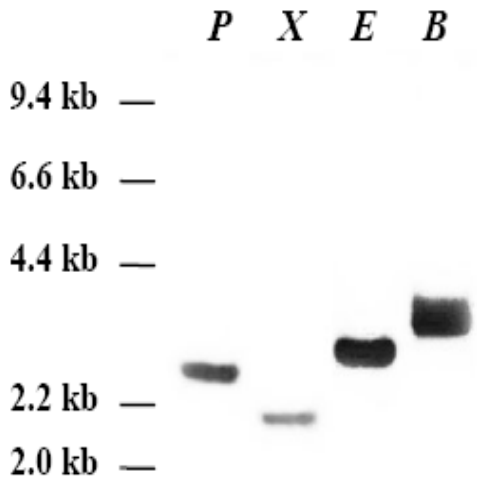

Figure 4. Southern blot analysis of dihydroflavonol 4-reductase in *Ascocenda* spp. DNA isolated from petals was digested separately with *Pst*I, *Xba*I, *Eco*RI and *Bam*HI, electrophoresed on 0.7% agarose gel and blotted onto nylon membrane. An internal fragment of the *AscoDFR* gene amplified with the primer AsDFR-F1 and reverse AsDFR-R1 was used as a probe.

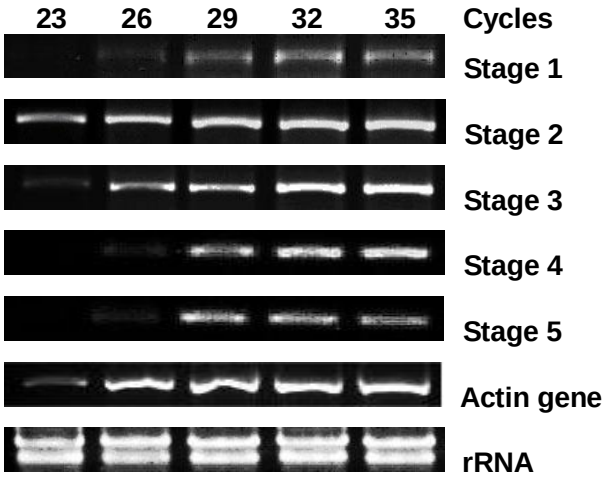

Figure 5. RT-PCR analysis of the *AscoDFR* gene expression in the different developmental stages of flower. Total RNAs were prepared from orchid flowers at stage 1 to 5 and subjected to RT-PCR analysis with primers specific for the *AscoDFR* as described in materials and methods. Actin gene was used as an internal control. The numbers above the lanes represent the number of PCR cycles. Ethidium bromide-stained rRNA was used as a control to normalize the amount of total RNAs (10 μg).

down-regulated and the genes downstream of the *DFR* may be activated. The transcription level of the *AscoDFR* at flowers stage 2 and 3 was approximately three times higher than those observed at the flowers stage 1 (unopened bud with ca. 0.5 to 1.0 cm), stage 4 (partially opened flower) and stage 5 (fully opened flower). In view of the fact that flowers of *Ascocenda* spp. are rich with pelargonidin glucoside, thus it is not unexpected to detect an abundance of *DFR* transcripts in these organs. It should be concluded from these findings that the *AscoDFR* was expressed throughout flower development and its transcription was activated in flower bud prior to anthesis, since the expression of this gene was firstly detected at flower stage 1. Our findings are consistent with those of Farzad et al. (2003) and Zhang et al. (2008) who observed the accumulation of *DFR* genes throughout the developmental stages of grape berry fruit and *V. cornuta* flower, respectively. How DFR is involved in flower development and colouring or what factor is regulates the DFR activity is of interest and will be clarified in the future.

Various expression levels of the *DFR* gene in the floral organs have been reported in several plants. In rose (*Rosa hybrida*), for example, the accumulation of *DFR* transcripts is found in petals, sepals, thorns, styles and stamens (Tanaka et al., 1995). In *Foesythia* × i*ntermedia*, the accumulation level of *DFR* transcripts is mostly abundant in petals and sepals, and its absence in anthers (Rosati et al., 1997). In *B. finlaysoniana*, the *DFR* gene was expressed in all purple coloured tissues including sepal, petal, column and lip (Liew et al., 1998). With respect to the present study, the expression of the *AscoDFR* was detected in all floral organs including sepal, petal, column and labellum, but the maximum expression was illustrated in sepal and petal (Figure 6). The accumulation level of the *AscoDFR* transcripts in sepal and petal was approximately 3-fold higher than those observed in column and labellum, which was consistent with the presence of pigment in these organs. In orchid, sepal and petal are known as anthocyanin-accumulating organs, while column and labellum are known as anthocyanin-less organs. Thus, the higher transcription level of *AscoDFR* found in sepal and petal compared to column and labellum may reflect a quantitative organ-specific regulation of this gene, like that observed in *F. x intermedia* (Rosati et al., 1997; Durbin et al., 2003). Whether the regulation of *AscoDFR* in sepal and petal is independent of regulation of the pathway in column and labellum needs to be clarified.

There is a diverse group of factors that have been described in many plant species that modulate differential expression of the *DFR* genes, for example, light and flower pollination (Farzad et al., 2003) and UV treatment (Himi and Noda, 2004). Whether the *AscoDFR* expression is regulated by these factors remains to be investigated.

In conclusion, the *Ascocenda DFR* gene was cloned and characterized in this study. It contained 1,056 bp of coding sequence, encoded a protein of 351 amino acid residues with the calculated molecular mass of 39.8 kDa and the predicted isoelectric point of 5.58. The highly

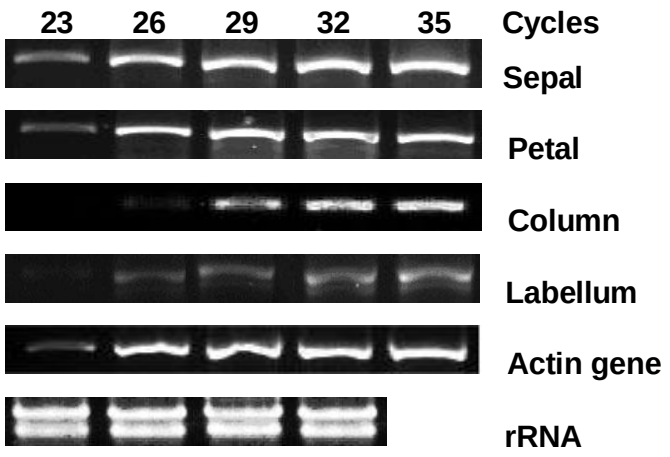

Figure 6. RT-PCR analysis of the *AscoDFR* gene expression in different floral organs. Total RNAs were prepared from sepal, petal, column and labellum of orchid flowers and subjected to RT-PCR analysis with primers specific for the *AscoDFR* as described in materials and methods. Actin gene was used as an internal control. The numbers above the lanes represent the number of PCR cycles. Ethidium bromide-stained rRNA was used as a control to normalize the amount of total RNAs (10 μg).

conserved regions among all *DFR* sequences such as a putative NADP binding region and the binding site of DHQ, DHM and DHK were present in *AscoDFR*. The amino acid sequence of the *Ascocenda DFR* gene product showed relatively high homology to those of *DFR* gene products from other orchids such as *Bromheadia*, *Dendrobium*, *Cymbidium* and *Oncidium*. It also showed high homology to the *DFR* gene products in other flowers such as *Lilium*, *Tilipa*, *Allium*, *Gentiana* and *Chrysanthenum.* The *AscoDFR* gene was highly expressed in flower at stage 2 and 3 of development as well as in the floral organs like sepal and petal. These data would be expected to be beneficial for future work such as modification of flower colour of orchid by using genetic transformation.

ACKNOWLEDGEMENTS

We gratefully acknowledge the Bangkok Green Co. Ltd., Thailand for providing the orchid flowers. This research was supported by the Thailand Research Fund (TRF) Master Research Grants (Grant no. MRG-WI525S037). The authors would like to thank the TRF for a grant to Wuttisak Kunu (W.K.).

REFERENCES

Aida R, Yoshida K, Kondo T, Kishimoto S, Shibata M (2000). Copigmentation gives bluer flowers on transgenic torenia plants with the antisense dihydroflavonol 4-reductase gene. Plant Sci. 160:49-56.

Durbin ML, Lundy KE, Morrell PL, Torres-Martinez CL, Clegg MT (2003). Genes that determine flower colour: the role of regulatory changes in the evolution of phenotypic adaptations. Mol. Phylo. Evol. 29:507-518.

Farzad M, Griesbach R, Hammond J, Weiss MR, Elmendorf HG (2003). Differential expression of three key anthocyanin biosynthetic genes in a colour-changing flower, *Viola cornuta* cv. Yesterday, Today and Tomorrow. Plant Sci. 165:1333-1342.

Himi E, Noda K (2004). Isolation and location of three homologous dihydroflavonol 4-reductase (*DFR*) genes of wheat and their tissue-dependent expression. J. Exp. Bot. 55:365-375.

Holton TA, Cornish EC (1995). Genetics and biochemistry of anthocyanin biosynthesis. The Plant Cell 7:1071-1083.

Johnson ET, Ryu S, Yi H, Shin B, Cheong H, Choi G (2001). Alternation of a signal amino acid changes the substrate specificity of dihydroxyflavonol 4-reductase. Plant J. 25:325-333.

Johnson ET, Yi H, Shin B, Oh BJ, Cheong H, Choi G (1999). *Cymbidium hybrida* dihydroflavonol 4-reductase does not efficiently reduce dihydrokaempferol to produce orange pelargonium-type anthocyanins. Plant J. 19:81-85.

Joshi CP (1987). Putative polyadenylation signals in nuclear genes of higher plants: A compilation and analysis. Nucle. Acids Res. 15:9627-9640.

Lacombe E, Hawkins S, van Doorsselaere J, Piquemal J, Goffner D, Poeydomenge O, Boudet AM, Grima-Pattensati J (1997). Cinnamyl CoA reductase, the first committed enzyme of the lignin branch biosynthetic pathway: Cloning, expression and phylogenetic relationships. Plant J. 11:429-441.

Lee WS, You JA, Chung H, Lee YH, Baek NI, Yoo JS, Park YD (2008). Molecular cloning and biochemical analysis of dihydroflavonol 4-reductase (DFR) from *Brassica rapa* ssp. *pekinesis* (Chinese cabbage) using a heterologous system. J. Plant Biol. 51:42-47.

Liew CF, Loh CS, Goh CJ, Lim SH (1998). The isolation, molecular characterization and expression of dihydroflavonol 4-reductase cDNA in the orchid, *Bromheadia finlaysoniana*. Plant Sci. 135:161-169.

Piero ARL, Puglist I, Petrone G (2006). Gene characterization, analysis of expression and *in vitro* synthesis of dihydroflavonol 4-reductase from *Citrus sinensis* (L.) Osbeck. Phytochem. 67: 684-695.

Reddy A, Britsch L, Salamini F, Saedler H, Rohde W (1987). The *A1* (anthocyanin-1) locus in *Zea mays* encodes dihydroquercetin reductase. Plant Sci. 52:7-13.

Rosati C, Cadic A, Duron M, Renou JP, Simoneau P (1997). Molecular cloning and expression analysis of dihydroflavonol 4-reductase gene in flower organs of *Forsythia x intermedia*. Plant Mol. Biol. 35:303-311.

Sambrook J, Russell DW (2001). Molecular cloning: A laboratory manual. 3rd Edn., Cold Spring Harbor Laboratory Press, New York.

Stich K, Eidenberger T, Wurst F, Forkmann G (1992). Enzymatic conversion of dihydroflavonols to flavan-3, 4-diols using flower extracts of *Dianthus caryophyllus* L. (carnation). Planta. 187:103-108.

Tanaka Y, Fukui Y, Fukuchi-Mizutani M, Holton TA, Hoggins E, Kusumi T (1995). Molecular cloning and characterization of *Rosa hybrida* dihydroxyflavonol 4-reductase gene. Plant Cell Physiol. 36:1023-1031.

Tanaka Y, Tsuda S, Kusumi T (1998). Metabolic engineering to modify flower colour. Plant Cell Physiol. 39:1119-1126.

Tanaka Y, Katsumoto Y, Brugliera F, Mason J (2005). Genetic engineering in floriculture. Plant Cell Tiss. Org. Cult. 80:1-24.

Xie DY, Jackson LA, Cooper JD, Ferreira D, Paiva NL (2004). Molecular and biochemical analysis of two cDNA clones encoding dihydroflavonol 4-reductase from *Medicago truncatula*. Plant Physiol. 134:979-994.

Zhang P, Wen PF, Wan SB, Wang W, Pan QH, Zhan JC, Huang WD (2008). Molecular cloning of dihydroflavonol 4-reductase gene from grape berry and preparation of an anti-DFR polyclonal antibody. Vitis 47:141-145.