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Molecular characterization and expression analysis of dihydroflavonol 4-reductase (DFR) gene in *Saussurea medusa*

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Abstract Dihydroflavonol 4-reductase (DFR), which catalyzes the reduction of dihydroflavonols to leucoanthocyanins, is a key enzyme in the biosynthesis of anthocyanidins, proanthocyanidins, and other flavonoids of importance in plant development and human nutrition. This study isolated a full length cDNA encoding DFR, designated as *SmDFR* (GenBank Accession No. EF600682), by screening a cDNA library from a red callus line of *Saussurea medusa*, which is an endangered, traditional Chinese medicinal plant with high pharmacological value. *SmDFR* was functionally expressed in yeast (*Saccharomyces cerevisiae*) to confirm that SmDFR can readily reduce dihydroquercetin (DHQ) and dihydrokampferol (DHK), but it could not reduce dihydromyricetin (DHM). The deduced SmDFR structure shared extensive sequence similarity with previously characterized plant DFRs and phylogenetic analysis showed that it belonged to the plant DFR super-family. SmDFR also possessed flavanone 4-reductase (FNR) activity and can catalyze the conversion of eridictyol to luteoforol. Real-time PCR analysis showed that the expression level of *SmDFR* was higher in flowers compared with both leaves and roots. This work greatly enhances our knowledge of flavonoid biosynthesis in *S. medusa* and marks a major advance that could facilitate future genetic modification of *S. medusa*.

Keywords Dihydroflavonol 4-reductase · DFR · Flavonoids · Expression analysis · *Saussurea medusa*

Abbreviations

ANS	Anthocyanidin synthase
BA	Benzyladenine
CHI	Chalcone isomerase
DHK	Dihydrokaempferol
DHM	Dihydromyricetin
DHQ	Dihydroquercetin
DFR	Dihydroflavonol 4-reductase
HPLC-MS	High performance liquid chromatography-mass spectrometry
F3H	Flavanone 3-hydroxylase
F3′H	Flavanonoid 3′ hydroxylase
FNR	Flavanone 4-reductase
NAA	Naphthaleneacetic Acid
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
ORF	Open reading frame
PAL	Phenylalanine ammonia-lyase
UTR	Untranslated region

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Introduction

Saussurea medusa grows in snowy mountains at altitudes between 3,500 and 4,500 meters [1, 2]. *S. medusa* is a very important, but endangered, traditional Chinese medicinal herb used for the treatment of rheumatoid arthritis, mountain sickness, irregular menstruation, and cardiovascular diseases [3–6]. Flavonoids, such as 4′,5,7-trihydroxy-3′, 6-dimethoxy flavone (jaceosidin), 4′,5,7-trihydroxy-3′, methoxy flavone (hispidulin), apigenin, rutin, and anthocyanin are all extracted from the above-ground parts of *S. medusa* and are known to have anti-cancer and anti-inflammation activities [5–8]. It was also suggested that this herb might extend human life [4]. Flavonoids are responsible for many of the medical applications of *S. medusa*, so the manipulation of flavonoid biosynthetic pathways in *S. medusa* cell cultures would provide a powerful alternative to harvesting this rare plant from nature. The great demand for this plant, coupled with its special habitat and long growth cycle (4–5 years to reach maturity), means it would be beneficial to improve the flavonoid content and availability of this plant using genetic engineering.

Dihydroflavonol 4-reductase (DFR) has long been known to contribute to the biosynthesis of the flavonoids, anthocyanidin and proanthocyanidin [9–12]. DFR uses NADPH as a cofactor to catalyze the reduction of dihydroflavonols to their respective leucoanthocyanidins and these are common precursors for anthocyanin and proanthocyanidin biosynthesis (Fig. 1). The colorless, unstable leucoanthocyanidins are immediate precursors in the synthesis of anthocyanins, which are the major water-soluble pigments found in flowers and fruits. They are also precursors of catechins and proanthocyanidins, which are involved in plant disease resistance and also influence the food and feed quality of plant products [10, 13–15]. DFR was previously identified and characterized in several plant species, mainly as a single gene, but occasionally as a small gene family with multiple genes [12, 16–18].

In some plants, DFR is reported to have broad substrate acceptance. DFR accepts dihydroflavonols as substrates, but it also reduces flavanone as flavanone 4-reductase (FNR) to produce 3-deoxyanthocyanidin [16, 19, 20]. The 3-deoxyflavonoid pathway has received considerable attention in recent years, mainly because of the properties of 3-deoxyflavonoids in conferring resistance to fungal and bacterial plant pathogens and the antioxidant capacity of these compounds [15, 20].

We have utilized *S. medusa* tissue cultures for the past few years to investigate the regulation of flavonoid biosynthesis and we successfully identified several genes, including, *PAL*, *CHI*, *F3′H*, *F3H,* and *ANS* [1, 2, 7, 21, 22]. The *DFR* gene is a key gene late in the flavonoid biosynthesis pathway, so its characterization would be beneficial to future genetic modification of *S. medusa*.

For the first time this study investigated the cloning and characterization of the *S. medusa DFR* gene (designated as *SmDFR*, GenBank Accession No. EF600682). Biochemical data indicated that SmDFR functioned as a DFR and that it possessed FNR activity. This study will be useful in enhancing our understanding of SmDFR and in the near future should help regulate this important step in the *S. medusa* flavonoid biosynthetic pathway at the molecular level.

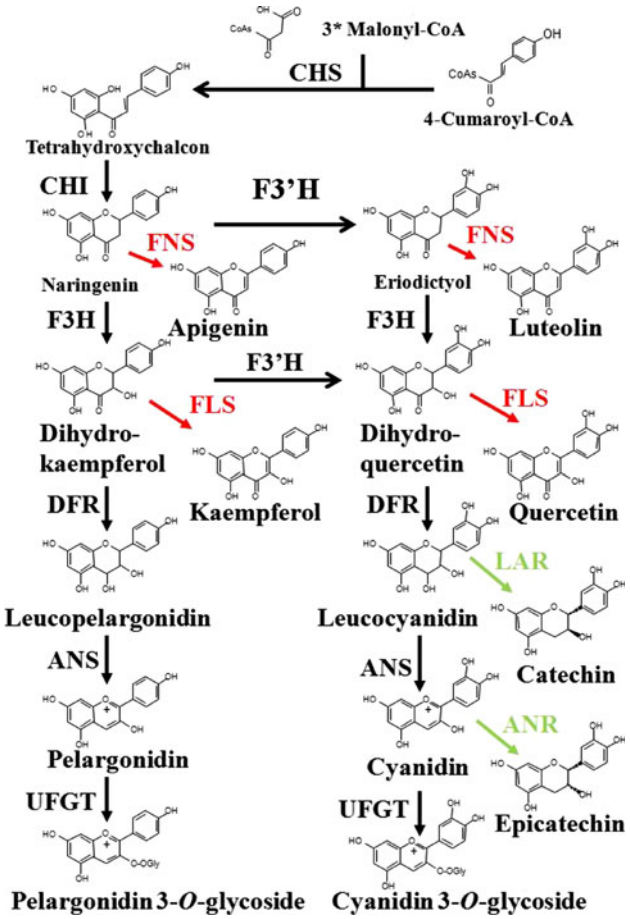

Fig. 1 The flavonoid biosynthetic pathway in *Saussurea medusa*. *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone 3-hydroxylase, *DFR* dihydroflavonol 4-reductase, *F3′H* flavanonoid 3′ hydroxylase, *FLS* flavonol synthase, *FNS* flavone synthase, *LAR* leucoanthocyanidin reductase, *ANR* anthocyanidin reductase, *ANS* anthocyanidin synthase, *UFGT, UDP* flavonoid glucosyl transferase. CHS, CHI, F3H, DFR, F3′H, ANS, and UFGT encoded gene sequences are available at the NCBI. FNS- and FLS-encoded genes were partially isolated in our lab. LAR- and ANR-encoded genes are not yet reported, but the catechin and epicatechin was detected by biochemical analysis

Materials and methods

Plant materials

A *S. medusa* red callus cell line [23] was maintained on MS medium supplemented with 3% sucrose, 2 mg l^{-1} NAA, 0.5 mg l^{-1} BA, and 0.6% agar, at 24 ± 1°C with a 16 h:8 h fluorescent light:dark photoperiod (60 μmol m^{-2} s^{-1}) and subcultured every 2 weeks. The callus was collected at defined times under specific conditions and immediately frozen in liquid nitrogen, then stored at –75°C until use [7].

Construction of cDNA library

Total RNA was isolated from 6-day-old calluses of *S. medusa* treated at 4°C for 24 h using Trizol Reagent (Gibco BRL, Grand Island, NY, USA), according to the manufacturer's instructions. The cDNA library was constructed as previously described [21].

Screening of the cDNA Library by PCR

The cDNA library was screened by PCR with degenerate primers (DFRg1: 5′-GAG AAT GAA GT (A/G) AT (A/C/T) AA (A/G) CC-3′; DFRg2: 5′-AAA ATA CAT CCA TCC (A/G/C/T)GT CAT-3′) that were designed based on the conserved regions of DFR from other plant species. Briefly, we mixed 0.5 ml of a fresh overnight bacterial culture grown in LB liquid medium and added phage containing the library. The mixture was incubated for 20 min at room temperature. To this 10 ml of LB medium was added and the mixture was dispensed with 100 μl $well^{-1}$ in a 96-well plate with an 8 × 12 matrix. The plate was sealed with polyester sealing tape and incubated for 5–6 h at 37°C, with shaking at 200 rpm, to amplify the phage in the subpool of the library. Then, we pipetted 10 μl of phage from each of the eight wells across a row and mixed them in a 1.5 ml test tube. We used 1 μl of the mixture as a template for the PCR test. One PCR-positive row was selected and we used 12 single wells as templates for the PCR test. One PCR-positive well was selected and we pipetted the culture into 0.5 ml of fresh overnight bacterial culture and repeated the previous steps.

Next we plated out one PCR-positive well onto solidified LB medium and incubated overnight at 37°C. Fifty single plaques were selected randomly for the PCR test. The PCR-positive single phage clones were converted into pTriplEx2 plasmids, according to the manufacturer's instructions (Clontech Laboratories), and sequenced using an ABI337 automated DNA sequencer (Applied Biosystems). Genomic DNA isolated from the calluses of *S. medusa* was amplified with specific primers (DFRf: 5′-CAACATGGTACAAGATTCTC-3′, DFRr: 5′-GTACA ATGAGACATAAATGT-3′) that were designed based on the cDNA sequence and the genomic DNA sequence of *SmDFR* obtained above.

Bioinformatics analysis

The bioinformatics software DNAMAN 4.0, DNAStar 5.0, and publicly available web tools were used for analysis of *SmDFR*. The nucleotide sequence, deduced amino acid sequence, and open reading frame (ORF) were analyzed, and sequence comparison was conducted via a database search with the BLAST program [National Center for Biotechnology Services, NCBI; http://www.ncbi.nlm.nih.gov]. Secondary and 3-D structures of SmDFR were predicted using online tools (http://ser-loopp.tc.cornell.edu/cbsu/loopp.htm, http://swissmodel.expasy.org/SWISSMODEL.html). SmDFR and other DFRs were retrieved from GenBank and aligned with CLUSTALW, using the default parameters. A phylogenetic tree was constructed using the CLUSTALW online program (http://www.ebi.ac.uk/Tools/clustalw).

Transcription profiling and real-time PCR

Quantitative real-time PCR was conducted to investigate the expression pattern of *SmDFR* in different tissues of *S. medusa*. We froze 200 mg samples of roots, leaves, and flowers using liquid nitrogen. The total RNA from these leaves was extracted using a Trizol kit (Invitrogen). A 1 μg sample of total RNA was treated with DNase to eliminate genomic DNA contamination. The presence of DNA residues was tested using 1 μl of total RNA as the template in a 25 μl standard PCR reaction with specific primers (58Sf: 5′-CGAAGCCTGCACAGCAGAAC-3′, 58Sr: 5′-GTGCATA GGAGAGCGATTCCCTCACG-3′) for the *S. medusa* 5.8S rRNA gene.

The PCR reaction was performed by denaturation at 94°C for 5 min, followed by 30 cycles of 45 s denaturation at 94°C, 45 s annealing at 55°C, and 90 s extension at 72°C. After a final extension at 72°C for 5 min, the amplified fragments were separated on a 1% agarose gel containing 0.5 μg ml^{-1} ethidium bromide in TAE buffer. The remaining RNA was reverse transcribed using oligo(dT)18 primers and a Primescript RT Reagent Kit (Takara Biotec), according to the manufacturer's instruction. The success of reverse transcription was verified by PCR of 1 μl cDNA using 58Sf and 58Sr primers, as previously described. Quantitative real-time PCR was performed using an iQTM SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with first-strand cDNA as the template on an iCycler iQ5 real-time PCR Detection System (Bio-Rad).

Determination of PCR efficiency and calculation of the mRNA transcript levels was conducted using Gene

Expression Macro Version 1.1 (Bio-Rad). The methodology of this macro was based on the algorithms outlined by Vandesompele et al. [24].

Transformation of *E. coli* and *Saccharomyces cerevisiae* with expression plasmids

Based on the sequence of *SmDFR* and the multiple cloning site of pYES2, primer were designed (*SmDFR-Yf*: 5′-TCC *AAG CTT* ACAACATGGTACAAGATTCTC-3′ and *SmDFR-Yr*: 5′-GCG *GAA TTC* AGTACAATGAGACAT AAATGT-3′) and used in the PCR amplification of *SmDFR*. cDNA of *S. medusa* was denatured at 94°C for 4 min, followed by 35 cycles of amplification (94°C and 55°C for 50 s, 72°C for 2 min) and 10 min at 72°C. The amplified product and the pYES2 vector were digested with *Hind*III and *Eco*RI, and purified. The PCR product was cloned into the pYES2 vector by ligation and the recombined product was transformed into competent cells of *E. coli* strain DH5∝ by the heat shock method. The bacteria was plated onto solidified LB medium (with 100 mg l^{-1} ampicillin) and incubated overnight at 37°C. Colonies were selected and cultured on liquid LB medium with antibiotics. The correct recombination of the plasmid was tested by restriction digestion. The plasmid isolated from the positive colony was sent to the Shanghai–Sangon Co. for sequence testing. The empty expression vector pYES2 and the expression MdDFR-pYES2 plasmids were used for the transformation of competent cells of *S. cerevisiae* strain INV Sc1 and subsequent expression. This process used the LiAC transformation method, according to the protocol provided by Invitrogen.

Heterologous expression in yeast (*S. cerevisiae*)

We inoculated 100 µl of the MdDFR-pYES2 plasmid and pYES2 plasmid transformed *S. cerevisiae* strain INV Sc1 suspension culture into 10 ml SC-Ura + 2% sucrose liquid media and incubated overnight at 28°C with vibration. The preculture was inoculated into 50 ml of new media (the 1:10 diluted pre-culture had an OD_{600} of 0.4) and incubated at 28°C at 120 rpm. The OD_{600} value was examined after ca. 10 h. When the OD_{600} reached 0.8–1.2 the culture was induced with 27 ml sterile galactose solution (200 g l^{-1}). The cells were harvested 12–15 h after induction when the OD_{600} of the 1:10 culture suspension measured between 0.6–1.2.

The culture was centrifuged for 5 min at 4000 rpm. The cell pellet was resuspended in 500 µl deionized water. The solution was centrifuged for 30 s at 4°C at 4000 rpm (all remaining steps were performed at 4°C). The supernatant was discarded and the pellet was resuspended in 500 µl of buffer: 100 mM H_3PO4 buffer (pH = 7.4), 1 mM EDTA (pH = 8.0), 5% glycerol, and 1 mM phenylmethanesulfonyl fluoride. The solution was centrifuged for 5 min and resuspended with the lysate to an OD_{600} of 50–100. Next, an equal volume of glass beads were added to the tube for cell disruption. The tube was briefly shaken for 30 s then placed on ice for 30 s. This procedure was repeated four times, until most of the cells were no longer intact. The mixture was centrifuged for 10 min at the highest speed setting. The clear supernatant was transferred into new centrifuge tubes and the protein concentration was measured using the Bradford protein assay, with bovine serum albumin (BSA) as the standard. The tube was frozen in liquid nitrogen and stored at –70°C.

DFR enzyme assays and product identification

An enzyme assay examined whether the cDNA clone exhibited DFR activity. The reaction was based on methods described in [25–27]. A mixture of 70 µl of prepared protein extract (0.15 µg $µl^{-1}$), 10 µl substrate (10 µg $µl^{-1}$ of substrate dissolved in methanol, i.e., dihydroquercetin, dihydromyricetin, and eridictyol), and 50 µl NADPH (10 mM, dissolved in100 mM Tris–HCl, pH = 7.5) was incubated at 30°C for four hours.

The reaction product was twice extracted with 400 µl ethyl acetate and the extracted solution was evaporated at 50°C until the sample was dried. The residue was dissolved in 500 µl of a mixture of *n*-BuOH-HCl (95:5, v/v) and incubated at 95°C for 30 min. The sample's metabolic profile was examined by HPLC–MS analysis. A Bruker Daltonics Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics, Germany) was connected with an Agilent 1100 HPLC system (Agilent Technologies, Germany) equipped with a quaternary pump and a variable wavelength detector for HPLC–MS analysis. Components were separated on a ZORBAX Eclipse XDB-C18 column (150 mm long × 4.6 mm internal diameter, particle size 5 µm) which was held at 30°C. The HPLC parameters ranged from 90% acetonitrile to 10% water (acidified with trifluoroacetic acid, pH = 3.0) to 10% acetonitrile to 90% acidic water in 20 min. The detection wavelength was 214 nm. The electrospray ionization voltage of the capillary was set at –4,000 V and the end plate at –500 V. Nitrogen was used as a drying gas at a temperature of 300°C with a flow rate of 10 l min^{-1}. Full scan mass spectra were measured in a scan range from 50 to 1,000 *m/z* with a scan resolution of 13,000 *m/z* s^{-1}, until the ICC target reached 20,000 or 200 ms. Tandem mass spectrometry was conducted using helium as the collision gas (3.56×10^{-6} mbar) with the collision voltage set at 1 V. Spectra were acquired in the positive and negative ionization modes. Data analysis was performed using Data Analysis 5.2 software (Bruker Daltonics). Metabolites were

identified based on their retention times, mass spectra, and product ion spectra when compared with the data from an authentic reference material. Compound signals were integrated in their $[M + H]^+$, $[M - H]^-$ or the $[M + HCOO]^-$ ion traces. Induction factors were calculated from signal intensities. Standards for dihydroquercetin, dihydrokaempferol, dihydromyricetin, and eriodictyol were purchased from Polyphenols Laboratories AS (Hanaveien 4-6, 4327 Sandnes, Norway).

Results

Cloning of the full-length cDNA of *SmDFR*

The degenerate primers, DFRg1 and DFRg2, were designed based on the sequences of the conserved regions of *DFR* from other plant species and used for screening the cDNA library of *S. medusa* [21]. A PCR-positive phage clone was obtained after cDNA library screening and this clones was sequenced using an ABI337 automated DNA sequencer (Applied Biosystems). An isolated cDNA encoding *DFR* was designated as *SmDFR* (Gen- Bank Accession No. EF600682). The 1166 bp full-length cDNA of *SmDFR* contained a 1029 bp ORF with a stop codon of TGA and encoded a protein of 342 amino acids. The calculated molecular weight of SmDFR was approximately 38.6 kDa, which is very similar to previously reported plant DFRs [18, 20, 28].

Bioinformatic analysis of *SmDFR*

The cDNA of *SmDFR* was analyzed using DNAMAN 4.0 and ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the results showed that the *SmDFR* cDNA contained a 60 bp (1–60 bp) 5′-untranslated region, a 77 bp (1090-1166 bp) 3′-untranslated region, and a 1029 bp (61–1089 bp) ORF that encoded 342 amino acid residues.

The full-length genomic DNA of *SmDFR* was 1871 bp (from the start codon ATG to stop codon TGA). Based on the intron/exon clip "GT-AG" rule and the comparative analysis of DNA sequences with the cDNA, we identified six exons that were separated by five introns. The first exon included a 181 bp-long 5′-UTR and the ATG start codon, while the sixth exon included a 265 bp long 3′-UTR and the termination codon TGA (Fig. S1).

The sequence alignment and BLASTN analysis against the GenBank database (http://www.ncbi.nih.gov) showed that *SmDFR* shares high homology with the *DFR*s from other plant of the Asteraceae family, including, *Centaurea maculosa* (*CmDFR*, 91%), *Callistephus chinensis* (*CcDFR*, 87%), *Gerbera hybrid* cv. 'Terra Regina' (*GhDFR*, 85%), and *Chrysanthemum x morifolium* (*CmDFR*, 85%), which suggests that *SmDFR* belongs to the *DFR* superfamily. BLASTP analysis indicated that SmDFR amino acid sequence was 91, 87, 84, and 85% identical to CmDFR, CcDFR, GhDFR, and CmDFR, respectively.

Secondary structure prediction for the SmDFR protein was performed using the HNN package (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html). Hierarchical neural network analysis showed that the SmDFR protein was composed of 35.09% α-helix, 19.01% extended strand, and 45.91% random coil. The helix and random coil were interlaced and dominated the majority of the secondary structure.

The conserved regions of SmDFR were analyzed using the CDD software package (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The results showed that SmDFR contained two typical conserved domains pfam01073 and COG0451.

The 3D structure of SmDFR was predicted with Swiss-Model by sequence homology-based structural modeling (http://www.cn.expasy.org/swissmod/SWISS-MODEL.html). Molecular modeling results revealed that SmDFR has a Rossmann fold structure at the N terminal, which is very similar to the *Vitis vinifera* DFR. This domain binds with $NADP^+$ in a catalytic reaction [28].

The CLUSTALW online program (http://www.ebi.ac.uk/Tools/clustalw) was used to construct a phylogenetic tree based on the deduced amino acid sequences of SmDFR, and other DFRs from different plant species, to investigate the evolutionary relationships among DFRs. The analysis showed that SmDFR has its closest relationships with DFRs from other plant species of the Asteraceae family (Fig. 2).

SmDFR expression analysis in different *S. medusa* tissues

Quantitative real-time PCR analysis was conducted using total RNA isolated from different tissues, including, leaves, roots and flowers, as previously described. *SmDFR* expression was detected in all tested tissues, but expression levels in tissues were significantly different. The *SmDFR* expression level in flowers was about 8.31-fold higher compared with the leaves, while the *SmDFR* expression level in the roots was much lower compared with the leaves (Fig. 3).

Functional expression of *SmDFR* in yeast, and in vitro biochemical characterization

To confirm the function of SmDFR, the complete ORF was cloned into the yeast expression vector pYES2. The SmDFR-pYES2 plasmid was transferred into *S. cerevisiae*

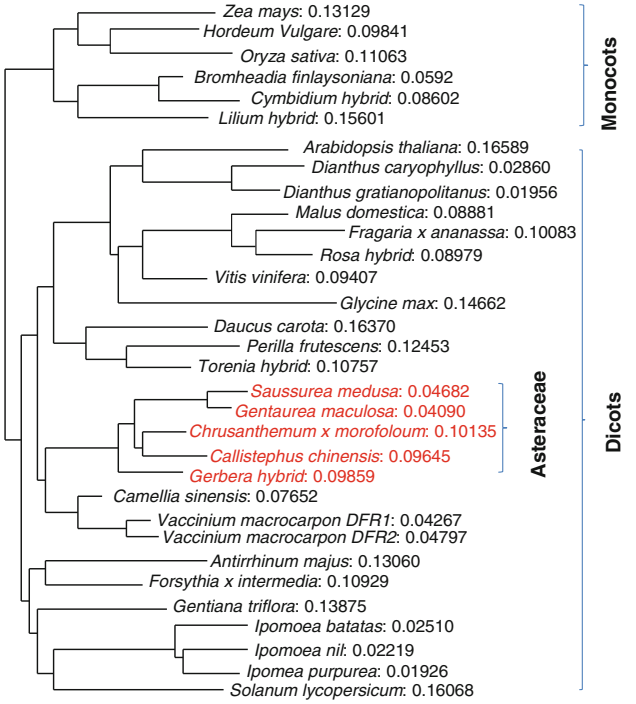

Fig. 2 Phylogenetic tree analysis of DFRs constructed from selected plant species using the CLUSTALW program. Dihydroflavonol 4-reductase amino acid sequences from the following species were used in the phylogenetic analysis: *Antirrhinum majus* (X15536); *Arabidopsis thaliana* (AB033294); *Bromhedia finlaysoniana* (AF007096); *Callistephus chinensis* (Z67981); *Camellia sinensis* (AB018685); *Centaurea maculosa* (FJ376591); *Chrysanthemum x morifolium* (GU324979); *Cymbidium hybrid* (AF017451); *Daucus carota* (AF184271); *Dianthus caryophyllus* (Z67983); *Dianthus gratianopolitanus* (AF291097); *Forsythia x intermedia* (Y09127); *Fragraria x ananassa* (AF029685); *Gentiana triflora* (D85185); *Gerbera hybrida* (Z17221); *Glycine max* (AF167556); *Hordeum vulgare* (S69616); *Ipomea batatas* (AB019243); *Ipomea nil* (AB006792); *Ipomea purpurea* (AB018438); *Lilium hybrid* (AB058641); *Lycopersicon esculentum* (Z18277); *Malus domestica* (AF117268); *Oryza sativa* (AB003495); *Perilla frutescens* (AB002817); *Petunia x hybrida* (AF233639); *Rosa hybrida* (D85102); *Torenia hybrida* (AB012924); *Vitis vinifera* (X75964); *Zea mays* (EU975645)

strain INV Sc1 and the enzyme was isolated, as previously described. The SmDFR enzymatic assay was performed using dihydrokaempferol (DHK), dihydromyricetin (DHM), dihydroquercetin (DHQ), and eriodictyol as substrates and with NADPH as a cofactor.

The extent of conversion of each substrate was quantified by HPLC–MS. When dihydroquecetin was used as the substrate, a new peak #1 ($m/z = 287$) clearly appeared before the substrate peak #2 ($m/z = 305$) (Fig. 4a). Mass spectrometry analysis indicated that this peak corresponded to leucocyanidin. Another new peak #3 ($m/z = 343$) was also detected and mass spectrometry analysis of peak #3 showed that it was also a very pure ion peak, which corresponded with *O*-butylcyanidin. When dihykaempferol was used as the substrate, a new peak #1 ($m/z = 271$)

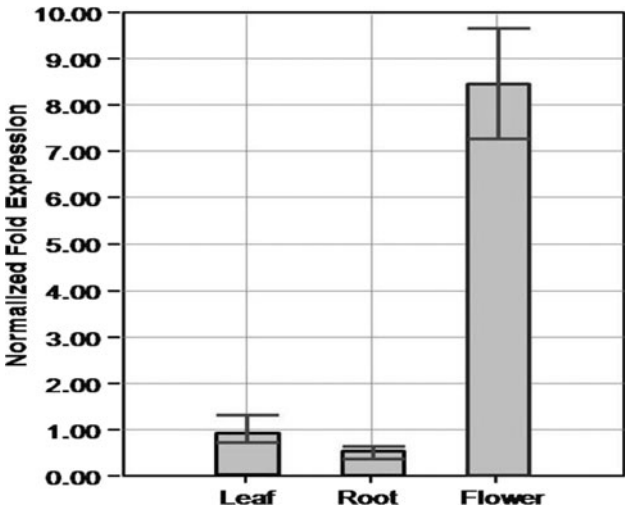

Fig. 3 mRNA expression levels of *DFR* genes in different *S. medusa* tissues. The values are expressed relative to a level of transcription in the leaf, which was set as one. Values are the means of three replicates ±SE

appeared before the substrate peak #2 ($m/z = 289$) (Fig. 4b). Mass spectrometry analysis indicated that the new peak corresponded to leucopelargonidin. Another new peak #3 ($m/z = 327$) was also detected, which corresponded to *O*-butylpelargonidin. This demonstrated that the SmDFR can catalyze dihydrokaempferol to generate leucopelargonidin. In contrast, the control solution catalyzed by the empty vector-induced protein did not produce these new peaks. This demonstrated that SmDFR can catalyze dihydroquercetin to produce leucocyanidin, and dihydrokaempferol to produce leucopelargonidin.

Surprisingly, no significant new peak was detected when dihymyricetin was used as the substrate (data not shown). This experiment was repeated five times and different reaction conditions were tested.

The flavanone, eriodictyol, was used as the substrate to test whether SmDFR had FNR activity and a new peak #1 ($m/z = 271$) was clearly detected before the substrate peak #2 ($m/z = 289$) (Fig. 4c). Mass spectrometry analysis of this peak showed that it was a very pure ion peak. The new peak #2 corresponded to luteoforol. No peak further new peaks followed the substrate peak #2. In contrast, the solution catalyzed by the empty vector-induced protein did not produce any new peaks. This demonstrated that SmDFR has FNR activity and can catalyze eriodictyol to produce luteoforol.

Discussion

DFR is a key enzyme in the formation of plant pigments and antioxidant, antifungal, and antibacterial flavonoid

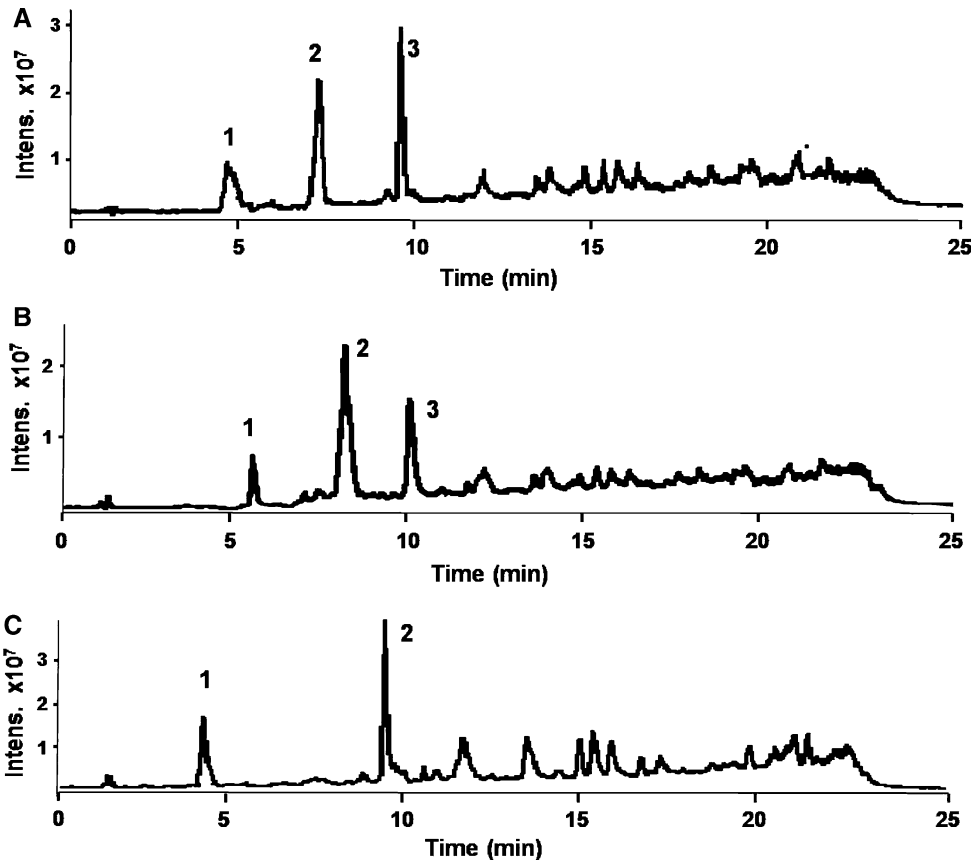

Fig. 4 Schematic HPLC–MS spectra for the enzyme assay of SmDFR-catalyzed reactions. **a** Reaction with dihydroquercetin used as the substrate: *peak 1* leucocyanidin; *peak 2* DHQ; *peak 3* *O*-butylcyanidin. **b** Reaction with dihydrokaempferol used as the substrate: *peak 1* leucopelargonidin; *peak 2* DHK; *peak 3* *O*-butylpelargonidin. **c** Reaction with eriodictyol used as the substrate; *peak 1* luteoforol; *peak 2* eriodictyol

compounds. DFR uses NADPH as a cofactor to catalyze the reduction of dihydroflavonols to leucoanthocyanidins, which are substrates for catechin and anthocyanidin biosynthesis. Catechins and an additional biosynthetic product, proanthocyanidin, have key roles in resistance to plant diseases [15, 25, 29]. Quercetin biosynthesis pathway takes a parallel pathway to leucoanthocyanidin biosynthesis. There are numerous accounts of the anti-cancer effects of quercetin in vivo. The risk of breast, lung, pancreatic, and stomach cancers is reduced in humans by the consumption of a large volume of quercetin in vegetables and fruits [9, 30, 31]. If the expression of DFR was blocked through RNA interference, then an alternative pathway to the production of quercetin would be induced and the anti-cancer properties of the plant would be enhanced [8, 32–34].

During the conversion of dihydroflavonol to leucoanthocyanidin, a hydroxy group is reduced making the polarity of leucoanthocyanidin greater than dihydroflavonol. Therefore, the new peak of leucoanthocyanidin should appear earlier than dihydroflavonol. Acidified butanol was added following the enzyme assay, which combined with leucoanthocyanidin to form butyl-leucoanthocyanidin during the heating process. The molecular weight of the new product was increased by 56 and a new peak was detected in the substrate by HPLC analysis [28]. The result was confirmed in our enzyme assay, where we used dihydroquercetin and dihydrokaempferol as substrates. During the conversion of eriodictyol to luteoforol only one new peak with $m/z = 271$ was produced and the peak with $m/z = 327$ was not detected. This might possibly be due to the lack of an active hydroxyl in position 3 [16].

Cheng et al. [7] studied the pigment composition of *S. medusa* using HPLC–MS and found that the main anthocyanidins in the red callus were cyanidin derivatives. Small amount of pelargonidin derivatives were also detected, but delphinidin derivatives were not detected in *S. medusa*. Data produced by the current study provide evidence that the isolated DFR enzyme actively uses dihydroquercetin (DHQ) and dihydrokaempferol (DHK) as substrates. The efficiency was higher for DHQ compared with DHK (Fig. 4) and these results were similar to those previously reported for the DFR found in *V. vinifera* [28]. The enzyme did not actively use dihydromyricetin as a substrate in the previous mentioned reaction conditions. Previous reports show that DHM is reduced by enzyme extracts from yeast expressing DFR from species naturally lacking delphinidin (*Gerbera, Matthiola, Rosa,* and *Dianthus*) to form leucodelphinidin [10, 20, 27]. This is the first report of a DFR lacking the activity to use dihydromyricetin as a substrate and this warrants further study.

The cloning and characterization of the *SmDFR* gene, which is a key enzyme in the late step of the flavonoid pathway, allowed us to elucidate the main flavonoid biosynthesis pathway in *S. medusa* (Fig. 1) for the first time. This will be very helpful for the further study of flavonoid biosynthesis in *S. medusa*.

In conclusion, we successfully cloned and characterized a new gene encoding *DFR* in *S. medusa,* which is involved in the biosynthesis of leucoanthocyanidin. Multiple alignments showed that the deduced *SmDFR* had high identity with other plant DFRs. The 3D model of SmDFR revealed a structure typical of DFRs. Expression analysis demonstrated that SmDFR possesses DFR and FNR activity. The cloning, characterization, and expression analysis of *SmDFR* provide novel insights into its role in the flavonoid biosynthesis pathway. This will provide a basis for improving the plant disease resistance and protecting human health by genetic engineering in *S. medusa* in the near future.

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