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Identification and characterisation of *DfCHS*, a chalcone synthase gene regulated by temperature and ultraviolet in *Dryopteris fragrans*

L. L. Sun, Y. Li, S. S. Li, X. J. Wu, B. Z. Hu and Y. Chang

Laboratory of Plant Research College of Life Sciences, Northeast Agricultural University, Harbin, 150030, China

Corresponding author: B.Z. HU and Y. Chang , Laboratory of Plant Research College of Life Sciences, Northeast Agricultural University, Harbin, 150030, China. Tel: 86 0451 55190410 E-mail: bzhu@neau.edu.cn (B. Z. Hu) / changying@neau.edu.cn (Y. Chang)

Abstract

Chalcone synthase (CHS) is an enzyme that catalyzes the first committed step in flavonoid biosynthesis, and its transcription level is regulated by light conditions. By using homology cloning and rapid amplification of cDNA ends, we cloned a chalcone synthase gene (*DfCHS*) from *Dryopteris fragrans* (L.) Schott. The full-length cDNA of *DfCHS* is 1,737 bp, with an open reading frame (ORF) of 1,122 bp (deposited in GenBank under Accession Number KF530802) encoding a predicted protein of 373 amino acids. The calculated molecular mass of DfCHS is 41.3 kDa. We studied the expression of *DfCHS* and total flavonoid contents in tissue culture seedlings cultured under the low temperature at 4ºC, high temperature at 35ºC and UV conditions, respectively. The results show that the expression of *DfCHS* are not the same, but all present rising trends, then flavonoid contents were increased. Overall, our results imply that the expression of *DfCHS* gene provide a certain theory basis in the status of evolution among ferns.

Key words: Chalcone synthase gene, *Dryopteris fragrans*, molecular cloning, gene expression, flavonoids.

Introduction

Dryopteris fragrans (L.) Schott is a species of the *Dryopteris* genus in the *Dryopteris* family. In China, this species is abundant in Wudalianchi (Heilongjiang Province), where a lava environment formed by volcanic eruptions can be found. It lives under lava at 30ºC to 60ºC, with a developed root system, but strong UV irradiation acquisition all year around. *D. fragrans* typically has a relatively long growth period and can endure low temperatures, even under –20ºC. Thus, it has become the focus of attention of local and international studies (1, 2). Additionally, *D. fragrans* has a complex chemical composition; a variety of its chemical compositions have been isolated, including isophthalics, terpenes and flavonoids (3, 4). Among these compounds, flavonoids have the most important role in plant growth, development, especially in the interaction between plants and environment, as it has many effects, including pathogen resistance, UV protection and defense (5-9).

Chalcone synthase (CHS) catalyzes the first committed step of flavonoids biosynthesis (7, 10). Proper illumination intensity or temperature condition can improve the transcription level of *CHS* gene, thereby changing the content of flavonoids in plant tissue (11-14). UV radiation induces a large amount transient expression of mRNA of *CHS* in *Petroselinum hortense*. The expression level of *CHS* in *Arabidopsis thaliana* is positively correlated with light intensity. Low temperature and relatively distinct temperature difference can induce its expression of *CHS* (15-18). The *CHS* catalyzes the production of a variety of secondary metabolites in bacteria, fungi, and plants (19). Researchers have cloned *CHS* genes or *CHS*-like genes from *Psilotum nudum* (20) in ferns, and even from *Marchantia paleacea* var. Diptera (21) in bryophytes. Thus far, about 700 *CHS*s and related gene sequences have been cloned; these genes are structurally similar, functionally related (22, 23). However, these genes have some differences in their expression and encoding products.

So far, few data have been reported about genes contributing to phenylpropanoid pathway in *D. fragrans*. In this study, we described the molecular cloning of a *DfCHS* gene from *D. fragrans* by rapid amplification of cDNA ends (RACE). Bioinformatics concerning the open reading frame (ORF), amino acid sequence, gene homology and phylogenetics were studied. Further, in order to explore the regulatory effect of *CHS* gene to plant defense response in *D. fragrans*, which lives in the special living environment, we determined the expression of *DfCHS* and total flavonoid contents at low temperatures, high temperatures, and by UV irradiation.

Materials and methods

Plant growth conditions and treatments

The sporophytes of *D. fragrans* were cultivated on the surface of improved 1/2 MS (Murashige and Skoog Basal Medium, PhytoTech, Shawnee Mission, USA) culture medium under a germ-free condition (25 ± 1ºC with a 12 h alternating photoperiod of light and darkness) until germination and growth of gametophytes. As soon as a fertilized gametophyte developed into a sporophyte, we performed secondary culture for the sporophyte on 1/2 MS culture medium.

The selected sporophytes were transferred in low-temperature conditions (4ºC), high temperature conditions (35ºC), and UV conditions for multiple treatments (0, 12, 24, 36, 48, 60, and 72 h for temperature treatments; 0, 3, 6, 9, 12, 18, and 24 h for UV treatments). After the different processing conditions, the samples

were placed directly in liquid nitrogen and stored at -80ºC.

RNA extraction

RNA was extracted from different samples of *D. fragrans* with RNAplant Plus Reagent (TIANGEN, Beijing, China) according to the manufacturer's protocols. RNA was quantified using a UV ultramicro spectrophotometer (Implen, München, Germany).

RACE

Single-strand cDNA was synthesized using L-A Taq DNA Polymerase (HaiGene, Harbin, China) according to the manufacturer's instructions. After RNase H treatment, the single-stranded cDNA mixture was used as a template for the polymerase chain reaction (PCR). One pair of specific primers (*DfCHS*-1 and *DfCHS*-2, Table 1) were designed according to the conserved sequences of *CHS* genes from closely related species and were used to amplify a *DfCHS* cDNA fragment of *D. fragrans*. The PCR reaction was tested using the following protocol: initial denaturing at 94ºC for 5 min, followed by 30 cycles of denaturing for 30 s at 94ºC, annealing for 30 s at 58ºC, and extension for 30 s at 72ºC, with a final extension of 10 min at 72ºC. After electrophoresis, the PCR products were recovered from the agarose gels with the use of a DNA gel extraction kit (HaiGene, Harbin, China), after which the fragments were ligated into the vector pMD18-T (TaKaRa, Dalian, China) and introduced into competent *Escherichia coli* strain DH5α cells. Recombinant plasmids recovered from positive colonies were sequenced to identify the core fragment.

The 5′ and 3′ RACE reactions used the SMART™ RACE cDNA amplification kit (Clontech, Mountain View, USA). Primers and nested primers are shown in Table 1. PCR was performed using the following PCR cycling conditions: 5 min denaturing step at 94ºC, 30 cycles of 55 s at 94ºC, 1 min at 56ºC for *CHS*-5-1 and *CHS*-5-2, or 1 min at 64ºC for *CHS*-3-1, followed by an additional step at 72ºC for 1 min, and finally 10 min at 72ºC for extension. The PCR products were gel-purified and sequenced as described above.

Bioinformatics analysis

Comparative and bioinformatic analyses were carried out online through the following websites: Open Reading Frame (ORF) finding was performed by the online program (www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequence comparison was conducted through database search using the BLAST program in the website of the NationalCenter for Biotechnology Information Website (http://www.ncbi.nlm.nih.gov). InterProscan (www.ebi.ac.uk/tools/pfa/iprscan) was used to analyze the protein domain/functional site, http://cn.expasy.org for *DfCHS*, http://www.cbs.dtu.dk/servicesSignalP/for SignalP 4.0, and http://www.cbs.dtu.dk/services/TargetP/ for TargetP V1.1. Phylogenetic analysis of DfCHS protein and *DfCHS* from other species was carried out by alignment with CLUSTAL X using default parameters. The protein sequences were aligned by BoxShade (http://www.ch.embnet.org/software/BOX_form.html). A phylogenetic tree was constructed by neighbor-joining method (24) using software MEGA version 4.0 (25).

Real-time PCR

The expression profiles of *DfCHS* were analyzed through real-time PCR. The primers (*DfCHS*-3 and *DfCHS*-4; Table 1) used are listed in Table 1. The 18s rRNA (18s-1 and 18s-2; Table 1) expression was chosen as a reference gene.

Real-time PCR assays (20 μL) were carried out using THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan) and multicolor real-time PCR detection system (Agilent, Santa Clara, USA). As an internal control, 18s ribosomal RNA was amplified, and all data were normalized to the 18s calculated threshold-cycle (Ct) level. To confirm the primer specificity, melting curve analysis of amplification products was performed at the end of each PCR reaction. The 18s rRNA expression was chosen as a reference gene. The PCR reaction was performed as follows: 95ºC for 1 min, followed by incubation for 15 s at 95ºC and denaturation for 35 s at 55ºC, and 40 cycles of elongation at 72ºC for 20 s. The result was analyzed according to the $2^{-\Delta\Delta CT}$ method (26).

Determination of total flavonoid

The total flavonoid contents in extracts was measured by a colorimetric assay (27, 28). The extract (10 mL) was added to a 50 mL flask, and then 5% $NaNO_2$ solution (0.5 mL) was added. After mixed well, the solution was allowed to stand for 6 min at room temperature; and 10% $Al(NO_3)_3$ solution (0.5 mL) was added to the flask, mixed well and kept for 5 min at room temperature. At last 4% NaOH solution (4.0 mL) was added, mixed well and kept for 15 min at room temperature. Absorbance was read on a TU-2401 UV-spectrophotometer (Shimidzu Co., Japan) at 510 nm, and the total flavonoid contents was estimated using calibration curves.

Results

Isolation and characterization of DfCHS

Using the specific primers (*DfCHS*-1 and *DfCHS*-2, Table 1), we obtained a putative 760 bp *DfCHS* gene fragment, and its nucleotide sequence was homogeneous to other known *CHS* genes by BLAST search in the NCBI website (http://www.ncbi.nlm.nih.gov/). Then, two fragments corresponding to the 5′ and 3′ ends of the *DfCHS* cDNA were amplified by RACE approach. The full-length cDNA of *DfCHS* was 1,737 bp, containing a 378 bp-3′ terminal UTR with a 2FE2S-FER-1 (PS00197) sequence CGTGGCATC (Fig. 1), 237 bp-5′ terminal UTR with a canonical polyadenylation signal sequence AATAAA, and a poly(A) tail (Fig. 1). The ORF of *DfCHS* sequence was 1,122 bp. It has been deposited in GenBank under Accession Number KF530802. Sequence analysis confirmed the clone to be a *DfCHS* gene.

The structure of DfCHS protein and Sequence analysis

The *DfCHS* gene contained a predicted 1,122 bp ORF encoding a 373 amino acid protein with a theoretical molecular mass of 41,293.4 Da. The average hydrophilicity was –0.195. The instability index was 39.31. The predicted amino acid sequence of DfCHS was highly similar to the reported plant CHS protein sequence. The DfCHS protein was further compared with homolo-

Table 1. Primers used for *DfCHS* gene cDNA PCR, RACE, and Real-time PCR

Oligo names	Length	Primer sequences
Primers for PCR		
DfCHS-1	29	5′-TTCTGCACCACCAGTGGGGTGGACATGCC-3′
DfCHS-2	35	5′-TGTTAGCCCTGGTCCAAACCCGAGAAGCAGATCCC-3′
Primers for 3′, 5′RACE		
DfCHS-5-1	24	5′-GAGGTTGGCTTGACATTCCACCTC-3′
DfCHS-5-2	21	5′-GGCACCATCTCCAAACAAAGC-3′
DfCHS-3-1	26	5′-GTGACGACAGGGGAAGGGTTTGAGTG-3′
Primers for Real-time PCR		
18S-1	23	5′-GCTTTCGCAGTAGTTCGTCTTTC-3′
18S-2	24	5′-TGGTCCTATTATGTTGGTCTTCGG-3′
DfCHS-3	26	5′-GGAAACATGTCGAGTGCGTGCGTGAT-3′
DfCHS-4	25	5′-ATCCCCACTCAAACCCTTCCCCTGT-3′

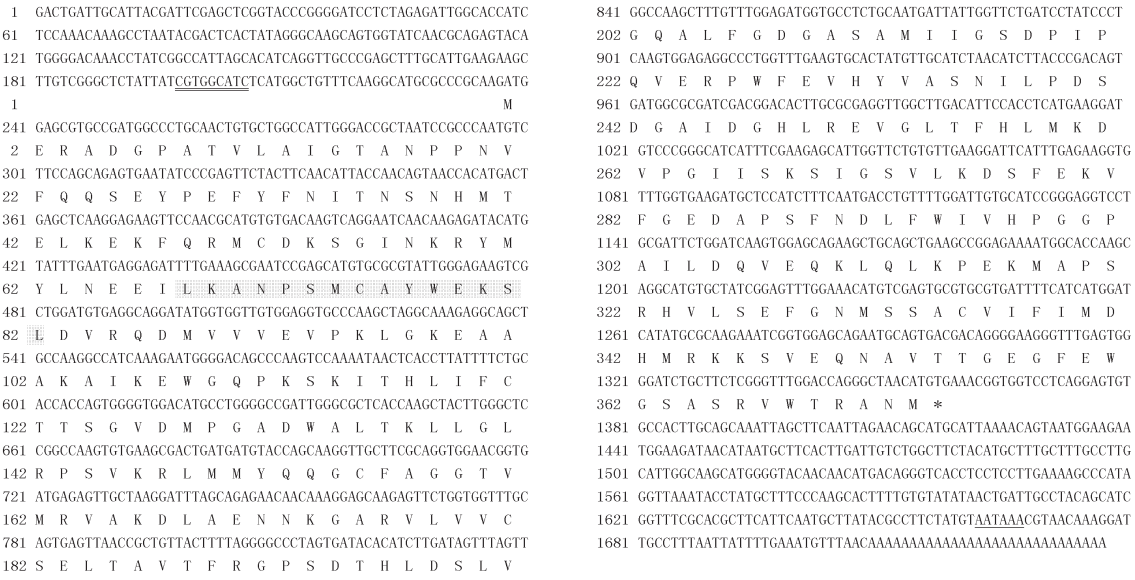

Figure 1. Nucleotide sequences and deduced amino acid sequences of *Dryopteris fragran*s (L.) Schott *DfCHS* cDNA. Asterisk (*) denotes the termination codon. One classical *CHS* signature motif is marked with a gray box; the canonical polyadenylation signal underlined and 2FE2S-FER-1 with doubeline, these nucleotide and deduced amino acid sequence data have been registered in GenBank (No. KF530802).

gous proteins by means of multiple sequence alignment and structure prediction analysis. The result showed that DfCHS contains the chalcone/stilbene synthase and the active site (147-163, RLMMYQQGCFAGGTVMR), which is a typical CHS protein tag (Fig. 1). In addition, the putative CHS protein may have five functional domains, namely, chalcone/stilbene synthase, N-terminal (IPR001099), polyketide synthase, type III (IPR011141), chalcone/stilbene synthase, C-terminal (IPR012328), thiolase-like, subgroup (IPR016038) and thiolase-like (IPR016039). Using the protein BLAST in NCBI BLAST, we found that this gene belongs to the superfamily of CHS. This result also further confirmed that the *DfCHS* that we obtained is truly part of the *CHS* gene of *D. fragrans*.

SignalP 4.0 and Target P V1.1 analyses showed that the DfCHS may not have a signal peptide. Thus, it was predicted to be a stable protein. BLAST analysis indicated that DfCHS is highly homologous to other plant CHS proteins (Fig. 2). The family signature of chalcone synthase (RLMMYQQGCFAGGTVLR) (29-31) is possessed in DfCHS. That is, 91% sequence similarity with the CHS sequence of *Ceratopteris thalictroides*, 80% sequence similarity with that of *Equisetum arvense*, and 77% sequence similarity with that of *Pseudotsuga menziesii*.

Phylogenetic tree analysis

Using the alignments of multiple amino acid sequences, *D. fragrans* DfCHS shared 75% to 91% similarity with other known CHSs. A phylogenetic tree was constructed to further identify the relationships between the DfCHS protein sequence and those of other plants that have already been obtained. As shown (Fig. 3), the phylogenetic tree is composed of four major groups: mosses, ferns, gymnosperms, and angiosperms. The relationship displayed in the phylogenic tree was generally in agreement with the traditional taxonomy of these species.

Real-time PCR analyses of DfCHS expression under different treatment conditions

To investigate the expression of *DfCHS* gene under different external stress conditions, we performed tests under different time points. For low temperature (4ºC), the results showed that *DfCHS* mRNA in response to the

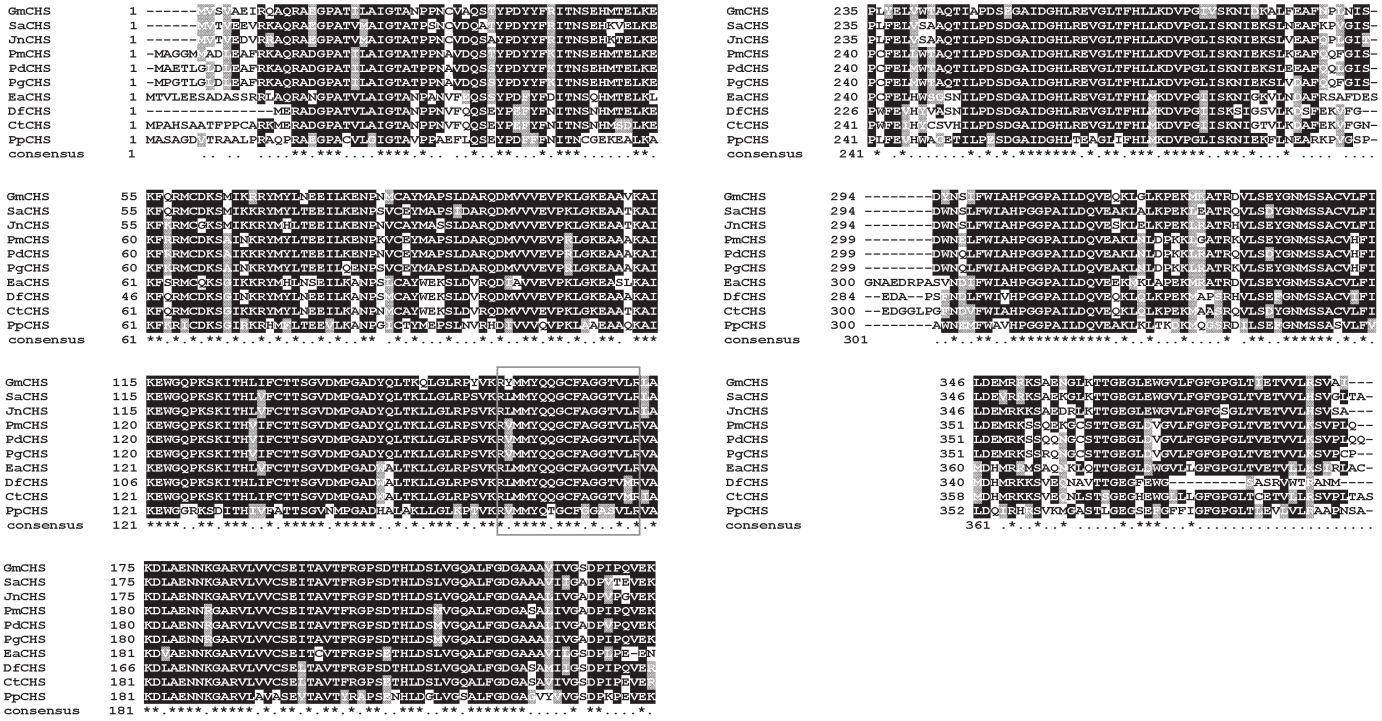

Figure 2. Alignment of DfCHS amino acid sequences with the amino acid sequences of CHS from other species. Alignment of deduced amino acid sequences of CHS from *Dryopteris fragran*s (L.) Schott (DfCHS, AHA85054.1) with those from *Glycine max* (GmCHS, AAO67373.1); *Sorbus aucuparia* (SaCHS, DQ286037.1); *Juglans nigra* (JnCHS, CAA64366.1); *Pseudotsuga menziesii* (PmCHS, ABD24227.1); *Pinus densiflora* (PdCHS, BAA94594.1); *Picea glauca* (PgCHS, AEN84260.1); *Equisetum arvense* (EaCHS, AB030004.1); *Ceratopteris thalictroides* (CtCHS, AFN02448.1); and *Physcomitrella patens* (PpCHS, ABB84527) are shown. The family signatures of chalcone synthase (RLMMYQQGCFAGGTVLR) is boxed.

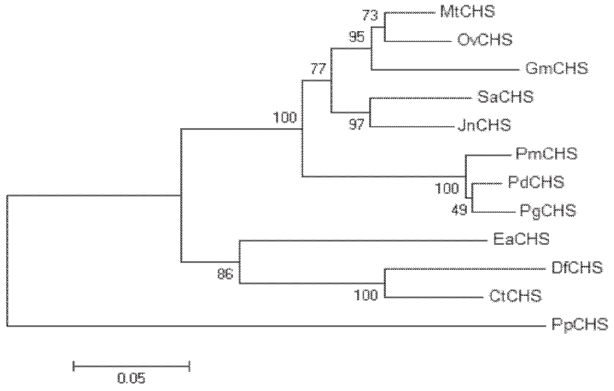

Figure 3. Phylogenetic tree illustrating the genetic relationships between CHS from *Dryopteris fragran*s (L.) Schott (DfCHS) and other plant CHSs. The tree was generated by MEGA 4.0 software using the neighbor-joining method following Clustal X. The scale bar indicates an evolutionary distance of 0.01 amino acid substitution per position in the sequence. Bootstrap values are indicated (1,000 replicates). GenBank IDs of CHS are *Medicago truncatula* (MtCHS, XP_003601647.1); *Onobrychis viciifolia* (OvCHS, HM204482.1);*Glycine max* (GmCHS, AAO67373.1); *Sorbus aucuparia* (SaCHS, DQ286037.1); *Juglans nigra* (JnCHS, CAA64366.1); *Pseudotsuga menziesii* (PmCHS, ABD24227.1); *Pinus densiflora* (PdCHS, BAA94594.1); *Picea glauca* (PgCHS, AEN84260.1); *Equisetum arvense* (EaCHS, AB030004.1); *Ceratopteris thalictroides* (CtCHS, AFN02448.1); and *Physcomitrella patens* (PpCHS, ABB84527).

length of treatment time. The expression of *DfCHS* gene was increased at first, but decreased eventually. The expression was highest in the 36 h treatment times (Fig. 4(A), P< 0.05). For high temperature (35ºC), the expression of *DfCHS* gene was most remarkable in the 24 h treatment times. The expression was lowest in the 60

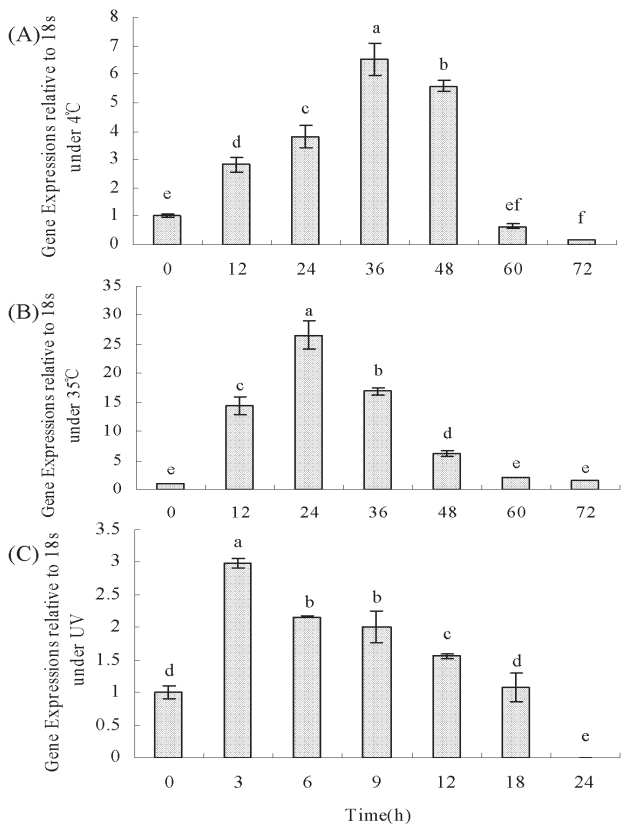

Figure 4. Quantitative real-time PCR analyses of *DfCHS1* expression in *Dryopteris fragran*s (L.) Schott under low temperature (4ºC) (A), high temperature (35ºC) (B), UV exposure (C). Expression of 18s rRNA was used as an internal control for real-time PCR. Each histogram represents the mean of three biological determinations. Means and standard errors of at least three independent experiments are reported. Different letters indicate significant difference (P<0.05, one-way ANOVA, followed by Duncan's test).

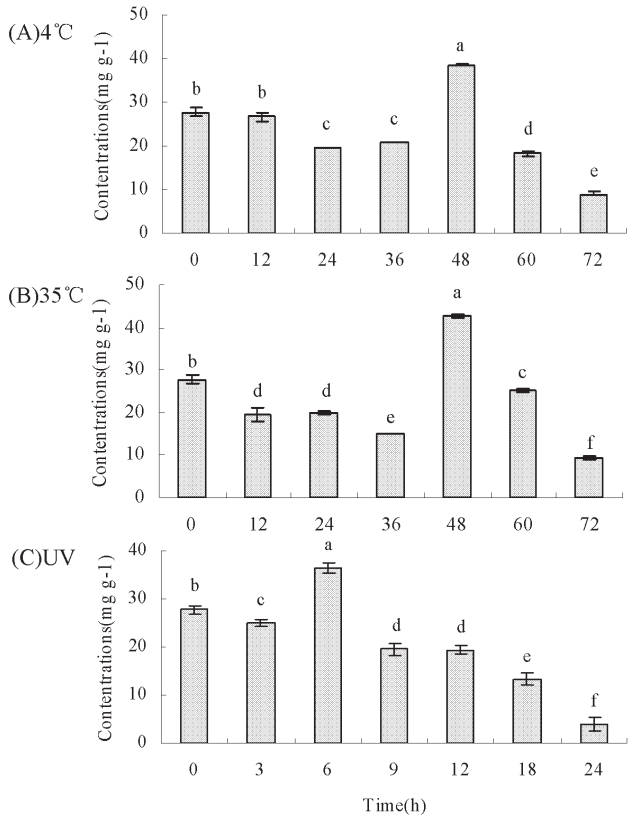

Figure 5. Total flavonoid content of *Dryopteris fragran*s (L.) Schott under low temperature (4ºC) (A), high temperature (35ºC) (B), UV exposure (C). The values and the error bars indicate the mean and standard error, respectively, from three independent measurements. Different letters indicate significant difference (P<0.05, one-way ANOVA, followed by Duncan's test).

and 72 h treatment times (Fig. 4(B), P< 0.05). Treatment with UV under different times changed the expression of the *DfCHS* gene. The expression of *DfCHS* gene was highest in the 3 h treatment times, then decreased gradually, almost no expression in 24 h (Fig. 4(C), P<0.05).

Total flavonoid contents of D. fragrans

The CHS is one of the key enzymes in flavonoid biosynthesis, by detecting the contents of flavonoid, to analyze the expression of *DfCHS* genes influence on flavonoid contents. As shown (Fig. 5), the total flavonoid contents were measured in different external stress conditions. We used the sample at 25ºC (0 h) as control. According to the calculation of standard curve, we obtained the contents as follows: the total flavonoid content of the control (25ºC) was 27.68 ± 0.49 mg ml^{-1}, flavonoids present rising trend under the bad outside environmental stimuli. Whereas, it was 38.47 ± 0.53 mg ml^{-1} for 48 h at 4ºC (Fig. 5(A), P<0.05); under 35ºC condition, the total flavonoid content was 42.70 ± 0.30 mg ml^{-1} at 48 h (Fig. 5(B), P<0.05); under UV condition, it was 36.51 ± 0.46 mg ml^{-1} at 6 h (Fig. 5(C), P<0.05), at other times, they were all less than the content in the control.

Discussion

The *CHS* gene exists in Bryophytes, Pteridophyta, Gymnosperm, and Angiosperm. Some of the reported *CHS* genes belong to a multigene family, and these genes are very conservative in structure (22). However, no previous studies have been reported describing the *CHS* gene in *D. fragrans*. In this study, we designed specific primers and cloned *DfCHS* gene fragments from sporophytes of *D. fragrans*. Furthermore, we acquired the 5′ and 3′ end sequences of this gene using the RACE method. We obtained the full-length cDNA sequence of the *CHS* gene called *DfCHS* (GenBank Accession Number KF530802) in *D. fragrans.*

Five putative functional domains can be found in the DfCHS protein, confirming that the *DfCHS* that we obtained is truly part of the *CHS* gene of *D. fragrans*. Furthermore, these functional domains are highly conserved in various plants. DfCHS contains the conserved active-site motif (147-163, RLMMYQQGCFAGGTVMR) of the CHS protein, which is a typical protein tag of chalcone/stilbene synthase. This finding suggests that the predicted amino acid sequence of *DfCHS* is accurate. Moreover, several studies have concluded that the sequences of the *CHS* genes are very highly conserved in numerous types of species. At the nucleotide level of the *CHS* member of different plants, the homology is mainly 90% higher with the same subfamily, and is more than 78% homologous with a different subfamily (32). The predicted amino acid sequence of the gene is highly consistent with the obtained plant CHS protein (75% to 91%). Based on multiple sequence alignments and phylogenetic analysis, DfCHS and other CHSs have strong connections in terms of structure and features. *CHS* gene sequences are relatively conserved, so they are crucial in phylogenetic analysis (33). Fern is located in the middle position of the phylogenetic tree of vascular plants; *D. fragrans* in fern is the transition group between angiosperm and bryophytes, so it has a crucial role in the phylogeny process.

CHS is the first key enzyme in the biosynthesis process of flavonoids, which is crucially important. However, CHS dose not regulate the flavonoids directly (8). We studied the expression of *DfCHS* and total flavonoid contents in tissue culture seedlings cultured under the same treatment condition, respectively. The results are that under low temperature at 4ºC (for 36 h), high temperature at 35ºC (for 24 h), and UV (for 3 h), *DfCHS* gene expression is at its highest. Accordingly, the total flavonoids content in *D. fragrans* increases to 38.47 ± 0.53 mg ml^{-1}, 42.70 ± 0.30 mg ml^{-1} and 36.51 ± 0.46 mg ml^{-1}, from 27.68 ± 0.49 mg ml^{-1} (25ºC in the control) under 4ºC (for 48 h), 35ºC (for 48 h) and UV (for 6 h) temperature stresses, respectively. As shown by the results, some changes have occurred for flavonoids under the conditions. But for different processing conditions, the changes in gene expression content of *DfCHS* are not the same. As the resistance to adverse environment by flavonoids is limited (8), the total contents of flavonoid showed a trend of decreasing followed by increasing then decreasing again. There are no correlation between the expression of *DfCHS* and total flavonoid contents. We found that even in the early stage of treatment, when the expression of *DfCHS* gene was high, the flavonoid contents had no corresponding rise. The fact that the gene presented increasing expression prior to downstream products might be due to genetic sensitive to external stimulus. In addition, flavonoid is not the first production generated by CHS enzyme. In the phenylalanine metabolic pathways, a phenylpropanoid CoA ester

under the action of CHS combining with malonyl-CoA molecules generated Chalcone ketones, again Chalcone ketones produces flavanone matter which have many biological activities under the action of chalcone flavanone isomerase, then the flavanone, different yellow ketone, flavanols, flavonol and other flavonoids are derived from the flavanone matter (34). So we could conclude that the increase of flavonoid contents needed a process of accumulation, finally it presented an out-of-synchronize condition between gene and the expression of the production. Under the harsh environmental stimuli that flavonoids present rising trend, prove it have the most important role in plant defense.

In conclusion, a *CHS* gene from the *D. fragrans* was identified and characterized for the first time. The expression of *CHS* gene provided certain theoretical basis in the status of fern evolution; it also presented a molecular basis for the regulation of flavonoids synthesis mechanisms and gene function orientation, Flavonoids have an important role in plant defense. Our study on *DfCHS* could facilitate further investigations, and can be applied to improve alkaloid contents in *D. fragrans* in the future (30). Further studies are needed to elucidate the relationships between the accumulation of flavonoids compounds and the expression of biosynthetic genes in *D. fragrans*. Currently, we are cloning more genes that are involved in the synthesis of flavonoids compounds.

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