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Identification and expression analysis of 4-Coumarate: Coenzyme A ligase gene family in Dryopteris Fragrans

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Abstract

4-Coumarate: coenzyme A ligase (4CL) catalyzes the conversion of hydroxycinnamates into corresponding CoA esters for biosynthesis of flavonoids and lignin. It has been widely studied in seed plants; however, it is poorly characterized in ferns. In this study, we identified 4CLs genes in ferns *D. fragrans* (L.) Schott (*Df4CL* gene) by rapid amplification of cDNA ends (RACE), and then investigated the expressions of the genes by real-time PCR, and determined total flavonoids and lignin contents. The results showed that four members of the *4CL* genes were found from this species, which named *Df4CL 1, 2, 3*, and *4* genes. Their full-length cDNA was sequenced. Also, our analyses showed that the amino acid sequences derived from these cDNAs exhibited similar conserved regions (Box I and Box II), and substrate-binding regions compared to 4CLs isolated from seed plants.At the same time, the developmental and stress-induced gene expression patterns showed that the changes on the expression levels of *Df4CL* genes affected the levels of flavonoids and lignin. In conclusion, we identified 4CLs genes in ferns *D. fragrans* and analyzed the expressions of these genes, and finally explored the relationship between the expressions of 4CLs and syntheses of flavonoids and lignin.

Key words: 4-coumarate: CoA ligase enzyme gene, *Dryopteris fragrans*, Phenylpropanoid metabolism, Molecular cloning, Gene expression, Flavonoids, Lignin, UV-light stress.

Introduction

Ferns are lineage of vascular plants that reproduce via spores instead of seeds. Understanding their development and function can provide important insights to plant genetic evolution, because they have typical characteristics of both ancestral and recent land plants (1). Ferns contain numerous secondary compounds that are interesting for studies, approximately one-fifth of them are produced by the phenylpropanoid metabolism pathway. In the pathway, the enzyme, 4-Coumarate: CoA ligase (4CL; EC 6.2.1.12), occupies a crucial position at the point of divergence from the general phenylpropanoid metabolism to several major branch pathways. Thus, 4CL is a key enzyme in the phenylpropanoid metabolic pathway for the biosynthesis of lignin and flavonoids that function in the production of plant aroma, fruit flavor, and color. These flavonoids also function as molecular signals, antimicrobial agents, flower pigments, antioxidants, tumor-fighting, and UV protectants (2-4), which make them important not only for plants in general, but also for many human uses. For example, many flavonoids have been used as health-promoting agents for thousands of years.

In fact, 4CL genes exist in plants as a family with multiple members. Based on the reports, the family has 4 members in *Glycine max* (*Gm4CL*) (5), five in *Oryza sativa* (*Os4CL*) (3, 6), four in *Physcomitrella patens* (*Pp4CL*) (7), and two in *Pinus taeda* (*Pt4CL*) (8). In *Arabidopsis thaliana* the family has 14 members, of those, only 4 genes had function and protein activity (9, 10).

Several groups have investigated the properties of 4CL enzymes (6, 7, 11, 12). In some species, such as *Solanum tuberosum, Petroselinum crispum,* and *Pinus taeda*, the cloned genes encode identical or nearly identical proteins that possess similar substrate affinities (13, 14). In some other species, such as *Populus tremuloides, Arabidopsis,* and *Glycine max,* the 4CL isoenzymes are distinct structurally and functionally (11, 12, 15, 16). These isoenzymes control the relative abundance of flavonoids and lignin during normal development (17). In terms of their functions reported, *At4CL1* and *At4CL2* gene along with *Gm4CL1* and *Gm4CL2* gene are involved mainly in the biosynthesis of lignin and other phenylpropanoids, whereas *At4CL3* gene and *Gm4CL3 , Gm4CL4* gene are involved mainly in the biosynthesis of flavonoids (5).

Phenylpropanoid metabolism is regulated primarily via transcriptional control of the corresponding genes (18). *4CL* gene expression is induced upon environmental stresses such as UV irradiation, under which the products of 4CL catalyzed the generation of phenylpropanoid derivatives that play an important protective role (18-23). The transcriptional activation of *4CL* genes has also been demonstrated with UV treatment of cultured cells from different plants including soybean, parsley and Arabidopsis as reported (3, 11).

The 4CL enzymes have been studied extensively in many species of seed plants; however, those of ferns remain poorly characterized. The object of our research was to characterize 4CL in the fern *Dryopteris fragrans* (L.) Schott, a deciduous perennial of the family Dryopteridaceae. It is well known for its medicinal uses in Northeast of China. Treatments with extracts prepared from the aerial parts of *D. fragrans* have been used in

traditional folk medicine for various dermatoses such as psoriasis, acne, and erythra. This fern is also known as an effective antibacterial, antioxidant, analgesic, antitumor, and antirheumatic agent (24-28). Dryofragin purified from *D. fragrans* is a phloroglucinol derivative, which has an anticancer activity for human breast cancer MCF-7 cells (29, 30).

In this study, it was the first time to report that *4CL*-genes were cloned from *D. fragrans* by rapid amplification of cDNA ends (RACE). Also we demonstrated that the existence of a family consisted of four genes in *D. fragrans*. In meantime, we examined the developmental and stress-induced gene expression patterns for the four *Df4CL* genes, and further determination of total flavonoids and lignin contents, finally explored the relationship between the expressions of 4CLs and syntheses of flavonoids and lignin.

Materials and methods

Plant materials and stress treatments

We collected spores of *D. fragrans* from Wudalianchi, Heilongjiang province in northeastern China (126°07′07″N, 48°42′38″E). The spores 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.

In order to study the developmental expression were collected mature gametophytes (MG) and various sporophyte organs of *D. fragrans* including root (Rt), petioles (Pe), and leaf (Le).For temperature treatment, the selected sporophytes were transferred in low temperature conditions (4ºC) and high temperature conditions (35ºC) under 0, 12, 24, 36, 48, 60 and 72 h. For UV-light treatment, the selected sporophytes were transferred in dark-adapted for 36 h and subsequently irradiated with UV-light at the different time (0, 3, 6, 9, 18 and 24 h) (11). After the different processing conditions, these samples were placed directly in liquid nitrogen and stored at-80ºC.

RNA isolation and cloning of full-length Df4CL cDNA using RACE

Total RNA was extracted from different samples of *D. fragrans* using the RNAplant Plus Reagent (TIANGEN, Beijing, China) according to the manufacturer's instructions. The quality of the RNA was determined using agarose gel electrophoresis, and the amount of RNA was quantified spectrophotometrically. Single-strand cDNA was synthesized using the HaiGene RNA PCR kit (HaiGene, Harbin, China) according to the manufacturer's instructions.

Transcriptome sequencing technology (31-33) was used to obtain the cDNA library of *D. fragrans*. By screening functional annotation of *4CL* genes from the library, and re-screening amino acid sequence with SSGTTGLPKGV (BOX I motif) and GEICIRG (BOX II motif) from the annotated 4CL genes, some special sequences were obtained, these special sequences were as the core fragments. After this, we amplified some of these fragments in order to obtain the complete *4CL* genes by rapid amplification of cDNA ends (RACE). Specific primers were designed according to the core fragments that we obtained. Df4CL 3′- and 5′-end cDNAs were isolated using a SMART RACE cDNA amplification kit (Clontech Laboratories Inc., USA). First-strand 3′-RACE-ready and 5′-RACE-ready cDNA samples from *D. fragrans* were prepared according to the manufacturer's protocol. The 3′-gene-specific primer *Df4CL* and 5′-gene-specific primers are listed in Table 1. 5' and 3' RACE PCR amplification was conducted using the specific primers for *Df4CL* and the UPM under the following conditions: 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 58 °C, and 2 min at 72 °C, with a final step of 10 min at 72 °C. After electrophoresis, the PCR products were recovered from the agarose gels with the use of a DNA gel extraction kit (HaiGene, Harbin, China), subcloned into the vector pMD18-T (TaKaRa, Dalian, China), transformed into the Escherichia coli strain DH5α, and then sequenced.

After aligning and assembling the sequences of the core fragment and the 3′- and 5′-RACE ends using the program Contig-Express (Vector NTI Suite 6.0), the full-length cDNA sequence of *Df4CL* was obtained and was used to design a pair of specific primers (Table 1). Full-length *Df4CL* cDNA was subsequently amplified by PCR with the specific primer pair using the following conditions: 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 58 °C, and 2 min at 72 °C, with a final extension for 10 min at 72 °C. The PCR products were gel-purifiedand sequenced as described above.

Bioinformatics analysis and phylogenetic analysis of the 4CLs

Comparative and bioinformatics 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 National Center 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. Amino acid sequences for the 4CL phylogenetic reconstruction were retrieved from GenBank (http://www.ncbi.nlm.nih.gov). The 4CL protein-coding sequences were aligned using CLUSTALW software implemented in MEGA5, and then manually edited. Neighbor-joining analyses (34) with 1,000 bootstrap replicates were performed using MEGA5 to reconstruct phylogenetic trees (35-36).

Gene expression analysis

The expression profiles of *Df4CL* were analyzed through real-time PCR. The primers used are listed in Table 1. The 18srRNA (18s-1 and 18s-2; Table 1) expression was chosen as a reference gene. Real-time PCR was performed on an ABI Prism 7500 sequence detector using the manufacturer's THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan). 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

Table 1. Oligonucleotide sequence of primers and their descriptions.

Primers	Sequence (5′ to 3′)	Description
4CL1-3RACE-F	TCTTCTACAAGAAGTTGCACG	3'-RACE specific primer
4CL1-5RACE-R	TATGCGAAGAGAGCAGAGGAGC	5'-RACE specific primer
4CL2-3RAC E-F	GGTCATGTGGCACGGTTGTTCG	3'-RACE specific primer
4CL2-5RACE-R	CGTAGCCACGGCACCACGCATA	5'-RACE specific primer
4CL3-3RACE-F	GCTGATGCACTCGGAAATCGTG	3'-RACE specific primer
4CL3-5RACE-R	CAGATTTCACTCGGGACGCATA	5'-RACE specific primer
4CL4-3RACE-F	CCTCAGAGTGTACTCAGAGAAGT	3'-RACE specific primer
4CL4-5RACE-R	AGCGATGGAGTTAGGGCAAAGG	5'-RACE specific primer
4CL1-ORF-F	TAGAGCGTCCATGGAGAGCCCTCCG	ORF forward primer
4CL1-ORF-R	AACAAATGAATAGCAATAAATAATGCCA	ORF reverse primer
4CL2-ORF-F	GGCTGAGGTCCTTCCCTCCTCTG	ORF forward primer
4CL2-ORF-R	ACTAGCGCTATTTGATTTTCTTAATGC	ORF reverse primer
4CL3-ORF-F	ATGGCAACCCCGAGCTTCGACCCG	ORF forward primer
4CL3-ORF-R	TTTGGATTGTGAATTATGTGTAAGC	ORF reverse primer
4CL4-ORF-F	ATGGCTGCCATAATCGACCCGAGGA	ORF forward primer
4CL4-ORF-R	CAATTTGGACATGGCAGCAGATACAAGCTC	ORF reverse primer
4CL1-qPCR-F	GTGCGAAATGCGGAGGTGA	qPCR forward primer
4CL1-qPCR-R	ATGGCTGTATGGGACAAGAGGAT	qPCR reverse primer
4CL2-qPCR-F	CTCATCGAGCACAGCAACACTGAATTCAA	qPCR forward primer
4CL2-qPCR-R	CTGGTACAACAGGCTTACCCAAAGGAGTC	qPCR reverse primer
4CL3-qPCR-F	GGCTATGGCATGACAGAAACT	qPCR forward primer
4CL3-qPCR-R	TCTGGATTGTTAAGATACCCTTGC	qPCR reverse primer
4CL4-qPCR-F	AGCAAGCGTGAGCCTGTGAAG	qPCR forward primer
4CL4-qPCR-R	AGGGCAAGGAAGCGACAAATAA	qPCR reverse primer
18sRNA-qPCR-F	GCTTTCGCAGTAGTTCGTCTTTC	qPCR forward primer
18sRNA-qPCR-R	TGGTCCTATTATGTTGGTCTTCGG	qPCR reverse primer

amplification products was performed at the end of each PCR reaction. 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 60ºC, and 40 cycles of elongation at 72ºC for 20 s. The result was analyzed according to the 2-ΔΔCT method (37). In all experiments, three individual plants of each group were chosen for RNA extraction, and then extracted RNA was used for real-time PCR experiment, which was repeated three times. In real-time PCR, the data were analyzed using the Duncan's test of SPSS software.

Determination of total flavonoids and lignin contents

The total flavonoid content in extracts was measured by a colorimetric assay (38, 39). 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 standard curve with rutin standard.

Lignin extracted with acidic dioxane was investigated as a possible standard for quantitatively determining lignin content in plant samples using the spectrophotometric method employing acetyl bromide (40, 41). Briefly, 10 mg of isolated lignin was dissolved in 5.0 mL of dioxane and aliquots of 0.6 mL were pipetted into culture tubes, frozen in liquid N_2, and placed on a freezedrier overnight. To each tube 0.5 mL of 25% acetyl bromide in glacial acetic acid (HAcBr) was added (cautionary note on acetyl bromide: corrosive; causes burns; irritating to eyes and respiratory sypetiole; reacts violently with water; lachrymator).A blank was included to correct for reagent background absorbance. Tubes were tightly capped (Teflon lined caps) and put in a 50 °C water bath for 30 min. After cooling the samples, all tubes received 2.5 mL of acetic acid (HAc), 1.5 mL of 0.3 M NaOH, and 0.5 mL of 0.5 M hydroxylamine hydrochloride solution. Tubes were shaken and HAc was added to give a final volume of 10.0 mL. Solutions were read at 280 nm in a TU-2401 UV-spectrophotometer (Shimidzu Co., Japan). Standard curves were developed from duplicate sample series.

Results

Comparative analysis of the Df4CLgene family

Four full-length 4CL genes were obtained from *D. fragrans*. In accordance with the standard 4CL numbering sypetiole (42), these genes were named as *Df4CL1* (KF801576), *Df4CL2* (KF836752), *Df4CL3* (KF836753), and *Df4CL4* (KF836754). The nucleic acid sequence comparison analysis revealed the identity of among these four genes is 66.00% (Supplementary Fig.1). The predicted Df4CL1, Df4CL2, Df4CL3, and Df4CL4 protein contain 539, 565, 552, and 573 amino acids, respectively. Comparing the four amino acid sequences an identity of 56.69% (Supplementary Fig.2) were found. The characteristics of their nucleic acids and amino acids derived were listed in Table 2.The further amino acid sequence comparison analyses showed there were two conservative regions in the sequences between *D. fragrans* and other plants (Fig.1). They were SSGTTGLPKGV (BOX I motif) and GEICIRG (BOX II motif). Box I of Df4CL1 and Df4CL2 were identical to the reported motif SSGTTGLPKGV (43, 44). Box I of Df4CL3 and Df4CL4 were SSGTTGVSKGV, in which there were two varied amino acids (LP to VS). Box II of Df4CL1 and Df4CL2 were identical to the

Table 2. Characteristics of the four Df4CL nucleic acids and proteins.

Gene	4CL1	4CL2	4CL3	4CL4
Full-length(bp)	1976	2027	1898	1918
Open reading frame(bp)	1617	1695	1656	1719
5'-UTR	91	225	47	47
3'-UTR	265	103	192	149
Initiation codon	ATG	ATG	ATG	ATG
Termination codon	TAG	TAG	TGA	TAA
Polya tail (bp)	27	21	26	29
Amino acid residues	539	565	552	573
Accession number	KF801576	KF836752	KF836753	KF836754

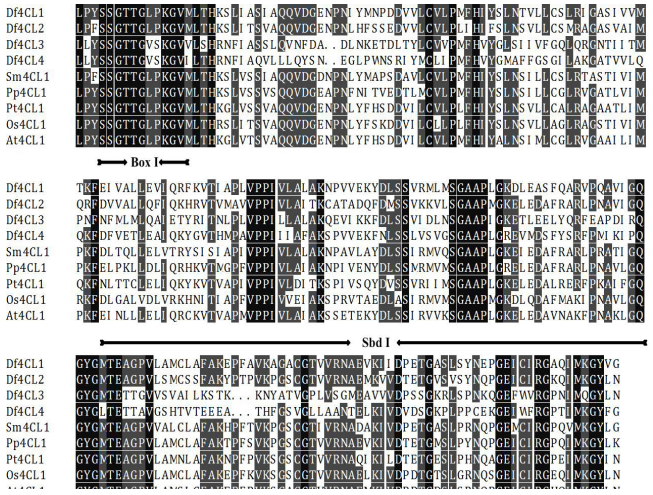

Figure 1. Comparison of amino acid sequences of different 4CLs. The image is reduced to the region flanked by the conserved peptide motifs Box I and Box II and substrate binding region Sbd I and Sbd II.

motif GEICIRG (45, 46), whereas Box II of Df4CL3 and Df4CL4 were displayed as a variant GEIWFRG.

Phylogenetic analysis of the Df4CL family

Phylogenetic reconstructions were performed to evaluate the relationships among *D. fragrans* 4CLs and most available amino acid sequences from the plant 4CL protein family (Fig. 2). The 4CLs from higher plants form two strongly supported clades districted from the 4CL of lower plant (moss and Selaginella moellendorffii) (47). However, the 4CL isoenzymes of *D. fragrans* were retrieved in three clades. Df4CL1 was located between *P. taeda* and *S. moellendorffii* appeared to be more closely related to *P. taeda* isoenzymes. Df4CL2 were positioned as a neighbor to mosses, the location is consistent with the more ancient evolutionary history of ferns. Whereas Df4CL3 and Df4CL4 were grouped in separated clusters.

Developmental expression of Df4CL genes and determination for the contents of total flavonoid and lignin

The developmental expression patterns of the four *Df4CL* genes are shown in Fig. 3. The results showed there were significant differences below: a) The expression levels of the four genes in mature gametophyte were significantly different from each other (Supplementary Table 1); b) In sporophytic petiole, root and leaf, the expression levels of the four genes were significantly different (Supplementary Table 2, Supplementary Table 3 and Supplementary Table 4).

The results for flavonoid and lignin were showed in Fig. 4(A). In the sporophyte stages, the highest flavonoid content was in the petiole, which was 31.68±0.96

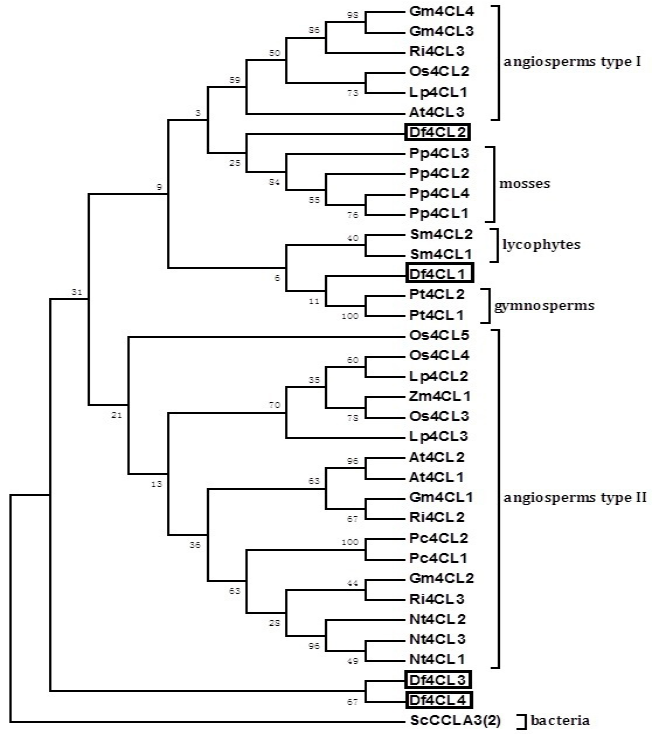

Figure 2. Phylogenetic tree illustrating the genetic relationships between 4CLs from D. fragrans and other plant 4CLs. The tree was generated by MEGA 5.0 software using the neighbor-joining method following Clustal X. The scale bar indicates an evolutionary distance of 0.01 amino acid substitution per position in the sequence. Bootstrap values are indicated (1,000 replicates). Accession no.NP_175579 (At4CL1, *Arabidopsis thaliana*), NP_188761(At4CL2, *A. thaliana*), NP_176686 (At4CL3, *A.thaliana*), AAL98709 (Gm4CL1, *G.max*), AAC97600(Gm4CL2,*G. max*), AAC97599(Gm4CL3,*G. max*), CAC36095(Gm4CL4,*G.max*), AAF37732(Lp4CL1, *L. perenne*), AAF37733(Lp4CL2, *L. perenne*), AAF37734(Lp-4CL3, *L. perenne*), AAA92668(Pt4CL1, *P. taeda*), AAA92669(Pt4CL2, *P.taeda*), XP_002969881(Sm4CL1, *S. moellendorffii*), XP_002979073(Sm4CL2, *S.moellendorffii*), ABV60447(Pp4CL1, *P. patens*), ABV60448(Pp4CL2, *P. patens*), ABV60449(Pp4CL3, *P. patens*), ABV60450(Pp4CL4, *P. patens*), AAF91310(Ri4CL1,*R. idaeus*), AAF91309 (Ri4CL2,*R. idaeus*), AAF91308 (Ri4CL3,*R. idaeus*), BAA07828(Nt4CL1,*N. tabacum*), AAB18637 (Nt4CL2,*N. tabacum*), AAB18638 (Nt4CL3,*N. tabacum*), CAA31696(Pc4CL1,*P. crispum*), CAA31697 (Pc4CL2,*P. crispum*), AAS67644(Zm4CL1,*Z. mays*), NP_001047819(Os4CL2, *O. sativa*), NP_001046069. (Os4CL3, *O. sativa*), NP_001058252 (Os4CL4, *O. sativa*), NP_001061935 (Os4CL5, *O. sativa*).

mg ml^{-1} (Fig. 4(A), $P<0.05$). The flavonoid content in leaves was 15.68±1.39 mg ml^{-1}. The flavonoids content was10.47±1.03 mg ml^{-1} (Fig. 4(A), $P<0.05$) in roots. While the lowest flavonoid content was in the gametophyte stage, which was 3.68±0.75 mg ml^{-1} (Fig. 4(A), $P<0.05$). The content of total lignin were

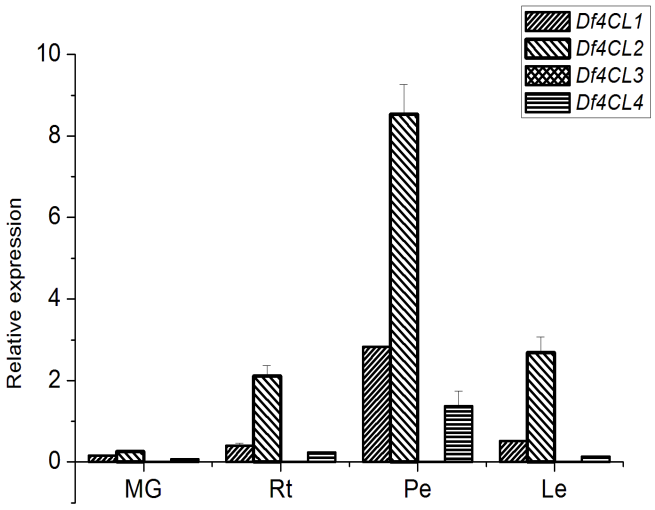

Figure 3. Expression of Df4CL genes in various organs of the fern *D. fragrans*. Total RNA from mature gametophytes (MG), roots (Rt), petioles (Pe) and leaves (Le) was used for real-time PCR analysis. 18s rRNA was used as an internal control for real-time PCR. Each histogram represents the mean of three 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).

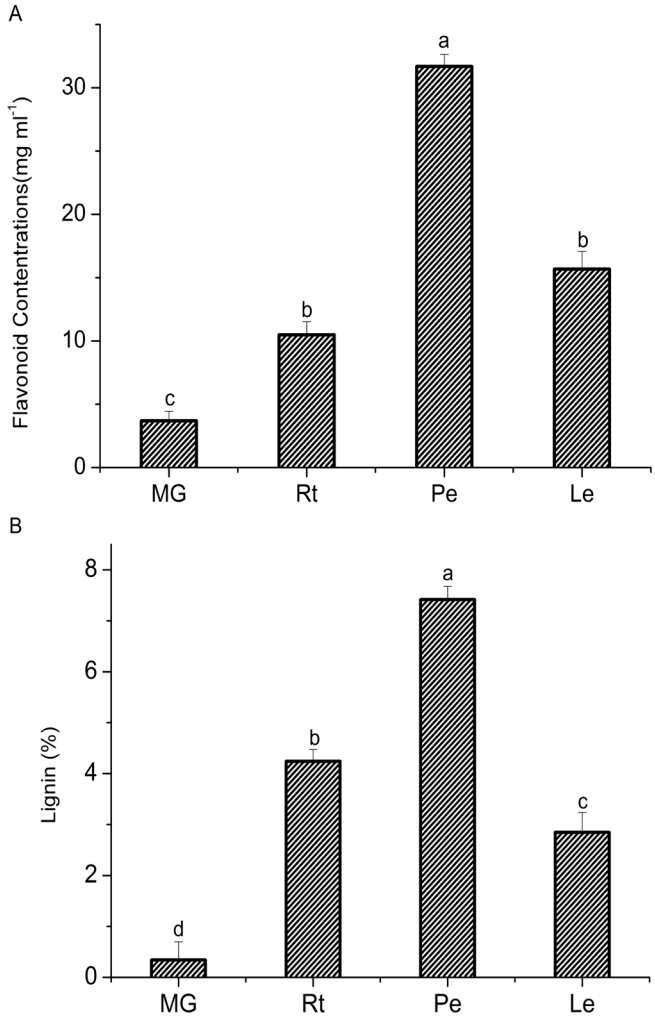

Figure 4. Total flavonoid (A) and lignin (B) content in various organs of *D.fragrans*. The values and the error bars indicate the mean and standard error, respectively, from three independent measurements. Different letters indicate significant difference (P<0.05, one-way ANOVA, followed by Duncan's test).

0.35±0.35%,4.24±0.23%, 7.42±0.26 % and 2.84±0.39% in mature gametophyte, roots, petioles, and leaves , respectively (Fig. 4(B), P<0.05)). 4CL genes were highly expressed on the locations, where flavonoid and lignin accumulated mostly, such as in the petioles.

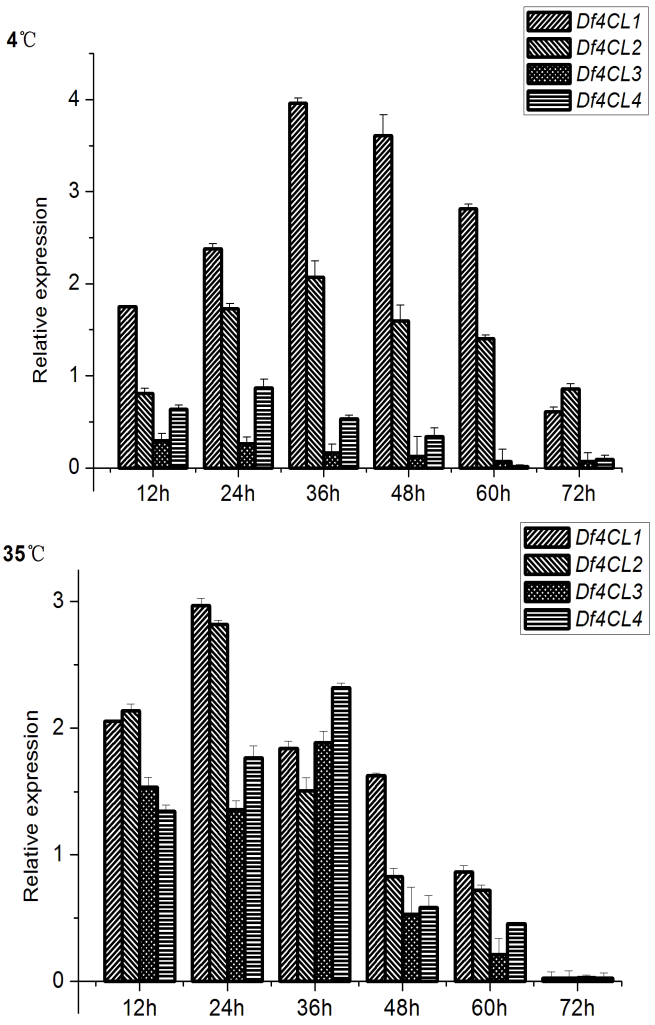

Figure 5. Real-time PCR analyses of Df4CLgenes expression level in *D.fragrans* under low temperature (4ºC), high temperature (35ºC). Expression of 18s rRNA was used as an internal control for real-time PCR. Each histogram represents the mean of three biological determinations. Means and standard errors of at least three independent experiments are reported. Different letters indicate significant difference (P<0.05, one-way ANOVA, followed by Duncan's test).

Temperature stress-induced expression changes of Df4CL genes and the changes of contents in total flavonoid and lignin

To investigate the expression changes of *Df4CL* gene under different external stress conditions, a time course for the expressions was tested. For low temperature (4ºC), the expression levels of *Df4CL* genes were increased at first, but decreased after. The expression levels of *Df4CL1*and *Df4CL2* gene were peaked at the 36 h (Fig.5, P< 0.05). The expression levels of *Df4CL3* and *Df4CL4* gene were peaked at the 24 h (Fig5, P< 0.05). For high temperature (35ºC), the expression levels of *Df4CL1*and *Df4CL2* gene were the highest in the 24 h. The expression levels of *Df4CL3* and *Df4CL4* gene were the highest in the 36 h. The expression levels were decreased quickly after peaking (Fig. 5, P< 0.05). No matter it was 4ºC or 35ºC, the expression levels of *Df-4CL1* gene and *Df4CL2* gene were always higher than the ones of *Df4CL3* gene and *Df4CL4* gene.

The tests for the contents of total flavonoids and

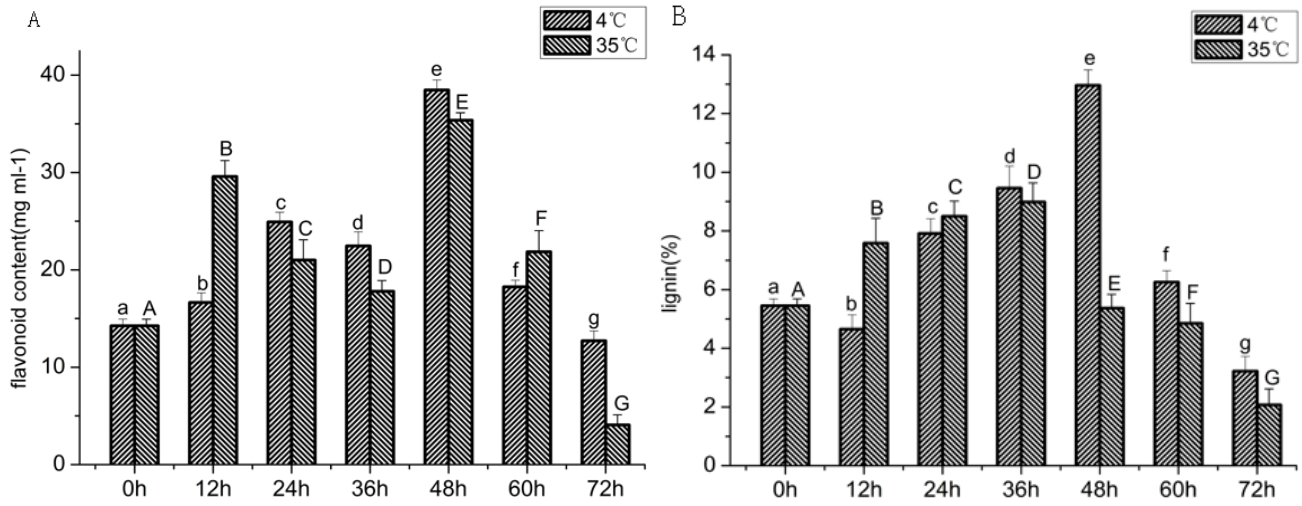

Figure 6. Total flavonoid and lignin content of *D. fragrans* under low temperature (4ºC), high temperature (35ºC). The values and the error bars indicate the mean and standard error, respectively, from three independent measurements. Different letters indicate significant difference (P<0.05, one-way ANOVA, followed by Duncan's test).

lignin were performed, and the results were shown in Fig.6. According to the standard curve, the contents were as follows: a) the content of flavonoids appeared a rising trend under the outside environmental stress. It peaked at 38.47 ± 1.03 mg ml^{-1} in 48 h under 4ºC (Fig. 6(A), P<0.05). Similarly, it peaked at 35.36 ± 0.76 mg ml^{-1} in 48 h under 35 ºC (Fig. 6(A), P<0.05). b) Regarding to the lignin, it peaked at 12.96 ± 0.53 % in 48 h under 4ºC (Fig. 6(B), P<0.05), and peaked at 8.98 ± 0.65 % in 36 h under 35 ºC (Fig. 6(B), P<0.05). Abiotic stresses, such as temperature, could activate *Df4CL* genes expression of *D. fragrans,* afterwards, changed contents of total flavonoid and lignin, and the changes on the contents of total flavonoids and total lignin lagged behind the expression of genes expression.

UV-light stress-induced expression changes of Df4CL genes and the changes of contents in total flavonoid and lignin

UV stress also changed the expression of the *Df4CL* genes (Fig.7). The expression levels were reached at the highest in 18 h for all 4 genes. However, the expression levels of *Df4CL3*gene and *Df4CL4* gene were significantly higher than the one of *Df4CL1* gene and *Df4CL2* gene.

Under UV light radiation stress, flavonoid content of *D. fragrans* increased significantly (Fig.8), and reached at the highest in 24 h (66.84 ± 1.24 mg ml^{-1} Fig. 8(A), P<0.05), while lignin content did not change much , and reached at the highest value in 6 h (6.84±0.49 % Fig. 8(B), P<0.05). In sum, UV-light stress-induced expression changes of *Df4CL* genes could change contents of total flavonoid and lignin, and the changes on the content of total flavonoids and total lignin lagged behind the expression of genes expression.

Discussion

Since the early evolution of land plants from primitive green algae, phenylpropanoid compounds have played an important role. In the biosynthesis of phenylpropanoids, 4-coumarate: CoA ligase has a pivotal role at the divergence point from general phenylpropanoid metabolism to several major branch pathways. Although

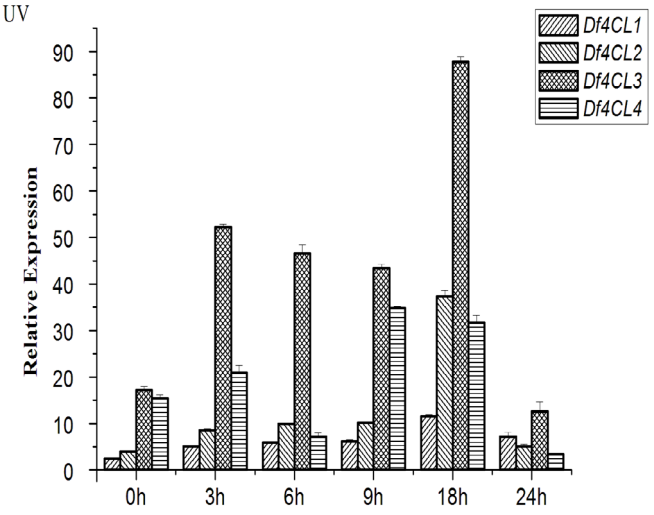

Figure 7. Real-time PCR analyses of Df4CLgenes expression level in *D.fragrans* under UV radiation. Expression of 18s rRNA was used as an internal control for real-time PCR. Each histogram represents the mean of three biological determinations. Means and standard errors of at least three independent experiments are reported. Different letters indicate significant difference (P<0.05, one-way ANOVA, followed by Duncan's test).

higher plant 4CLs has been extensively studied, little information is available on the enzymes from ferns.

We isolated 4 genes from *D. fragrans* and sequenced all of them for the first time. The comparison analysis of nucleic acid sequences indicated there was the identity of 66.00% among these four genes. It means that the four genes we obtained belonged to the same family. Also, the sequence analysis revealed that the gene family from *D. fragrans* shared 67%, 68%, 67%, and 62% identity with the 4CL gene family from *S. moellendorffii* , *P.taeda* ,*G.max* and *R.idaeus*, respectively. The further analysis on the the predicted amino acid sequence showed that the four genes had two highly-conserved peptide motifs, Box I (SSGTTGLPKGV) and Box II (GEICIRG) that existed in the other 4CL genes (43, 46, 48), which strongly supported that the four genes from *D. fragrans* could be the members of 4CL super family.

Phenylpropanoid metabolism is regulated primarily via transcriptional control of the corresponding genes. The accumulation of *4CL* mRNAs and the activity of their promoters vary depended on the tissues and cell

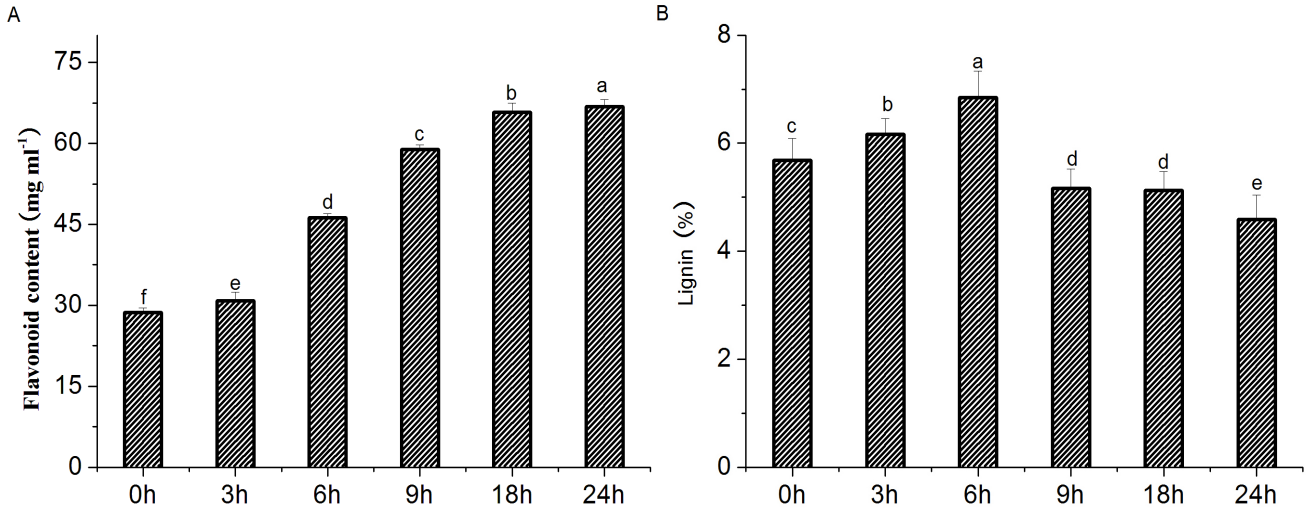

Figure 8. Total flavonoid and lignin content of *D. fragrans* under UV radiation. The values and the error bars indicate the mean and standard error, respectively, from three independent measurements. Different letters indicate significant difference ($P<0.05$, one-way ANOVA, followed by Duncan's test).

differentiation when certain cells become specialized for the biosynthesis of phenylpropanoid-derived compounds, such as lignin, flavonoids, and anthocyanin pigment (49, 50). Gene expression is also induced upon different organs and environmental stresses, such as wounding, pathogen attack, temperature and UV radiation, against which, phenylpropanoid compounds may have protective roles (51). Therefore, in this study, the expression patterns of the four Df4CL genes upon different organs and environmental stresses (temperature and UV radiation) were investigated.

As we known, the changes on the expression levels of genes would affect the contents of secondary metabolites. But for different processing conditions, the changes in gene expression content of *Df4CL* are not the same. As the resistance to adverse environment by flavonoids and lignin are limited, the total contents of flavonoid and lignin showed a trend of increasing then decreasing again. We found that the contents of the flavonoid and lignin were increased followed the increase of 4CL gene expressions, but not immediately, it took some times after the stimulations. So we propose that the increase of flavonoid and lignin contents needed a process of accumulation. Under the harsh environmental stimuli, the contents of flavonoids and lignin appeared to rise up, which suggest they play an important role in plant protection (18-23, 52). The study demonstrated that the 4CL genes from *D. fragrans* function as all the other 4CL genes under the normalization and the stimulations of temperature and UV.

Also, the analysis of phylogenetic tree indicated there was a closer relationship between the Df4CL isoenzymes and 4CL protein family of the higher plant. It implied that Df4CLs isoenzymes originated from a very early stage in the evolution of land plants. Although they are located in different locis in the phylogenetic tree, but the trend of the expression of gene was the same as the one of the accumulation of secondary metabolites basically. Our findings provided further evidence to support the importance of 4CLs in metabolism of phenylpropanoid pathway and evolutionary studies (53).

In conclusion, by the analysis of sequencing and functional study, we demonstrated that the genes we isplated from *D. fragrans* were the members of the 4CL gene family. Further studies are needed to elucidate the relationships between the accumulation of flavonoids and lignin compounds and the expression of the genes in *D. fragrans*.

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