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cDNA Cloning and Expression Analysis of the Chalcone Synthases (CHS) in *Osmanthus fragrans*

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Abstract

In the flavonoid biosynthesis pathway, Chalcone synthase (CHS) is involved in the formation of the pigment and has been shown to be a rate-limiting enzyme for the synthesis of flavonoids. In this study, a PCR approach was used to clone a Chalcone synthases cDNA from flower of sweet osmanthus "Chenghong Dangui" and it was designated as *OfCHS* (*O. fragrans*, CHS). The cDNA was 1383 bp long and a coding sequence (CDS) of 1173 bp encoding a polypeptide of 391 amino acids with an estimated molecular mass of 39.9 kDa. The theoretical isoelectric point was 6.23. Phylogenetic analysis demonstrated that OfCHS clustered with *Olea europaea*, *Solenostemon scutellarioides*, *Perilla frutescens*, *Antirrhinum majus* and *Digitalis lanata*. We also detected the expression of *OfCHS* in different tissues in "Dangui" and in two cultivars with varied coloration, "Zi Yingui" and "Chenghong Dangui" at different floral stages using quantitative real-time PCR. We observed that *OfCHS* transcript was higher in leaves than in petals in "Dangui". The transcripts of *OfCHS* in "Zi Yingui" petals were higher than those in "Dangui" at three stages especially at xianyan stage and there was no significant difference between the two cultivars in the full flowering stage. "Chenghong Dangui" has a relatively high anthocyanin content compared to "Zi Yingui". The relative amount of anthocyanin of "Chenghong Dangui" initially increases, and then decreases during the bloom period. However, the expression of CHS is the highest at the initial flowering stage. These data suggest that the *OfCHS* does not play a key role in the accumulation of total flavonoid in this cultivar. These data could contribute to explain the different accumulation of flavonoids in petals of the two cultivars.

Keywords

Osmanthus fragrans, Chalcone Synthases, Expression Analysis, Content of Anthocyanin

1. Introduction

Flower color is one of the most important characters of ornamental plants, and directly

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affects the ornamental value and economic value; creating with novelty colors has been the pursuit of plant breeders.

The flavonoids, a class of secondary plant metabolites, played the most important role in flower coloration of many flowering plants, increased and reduced anthocyanin content of petals and pericarp which are likely to make the color change [1] [2]. In plants, flavonoids are synthesized via the phenylpropanoid and polyketide pathway, which starts with the condensation of one molecule of CoA-ester of cinnamic acid or derivatives such as coumaric or ferulic acid, and three molecules of malonyl-CoA, yielding a naringen in chalcone as major product. A number of enzymes are involved in the flavonoids biosynthesis; genes encoding these enzymes have been isolated from many plants [3]. Chalcone synthases (CHSs), the most well known representatives of this family, provide the starting materials for a diverse set of metabolites (flavonoids) which have different and important roles in providing floral pigments, auxin protection and transport and stress resistance [4] [5] [6] [7].

Sweet osmanthus (*Osmanthus fragrans*) is one of the important ornamental plants in the Oleaceae family. Owing to their pleasant scent, and potential medicinal value, *Osmanthus fragrans* is widely distributed in China, Japan, Thailand, and India, and was introduced in Europe late in the 18th century [8]. So far, there are a total of 166 cultivars that have been identified and divided into four groups based on traditional, morphological and agronomic traits (e.g., flower color): *O. fragrans* Asiaticus Group, *O. fragrans* Albus Group, *O. fragrans* Luteus Group, and *O. fragrans* Aurantiacus Group [9].

Interestingly, flower color varies among the four groups and even at different stages of blossom development. The flower color in the Aurantiacus Group is mainly orange or orange-red, while in other three groups is ivory or yellow. Previous studies have shown that the major flower pigment compounds are flavonoids and carotenoids in sweet osmanthus [10]. However, there have been only few molecular studies of the anthocyanin biosynthesis pathway in sweet osmanthus. In this study, we cloned *CHS* cDNA from *O. fragrans* and analyzed CHS expression pattern of *OfCHS* in flowers and other tissues. We also detected the different expression of CHSin petals of Yingui and Dangui in different floral stages. These results provided basic information to study the function of *OfCHS* in *O. fragrans* flavonoids biosynthesis and metabolism.

2. Material and Methods

2.1. Plant Materials

Two varieties of *O. fragrans*, "Zi Yingui" and "Chenghong Dangui" used in this study were grew on the South campus of Shandong Agricultural University. Flowers from xianyan flowering stage (S1), initial flowering stage (S2) and full flowering stage (S3) [11] and mature leaves (L) were collected from sweet osmanthus trees, immediately frozen in liquid nitrogen and stored at −80˚C untilfurther used.

2.2. Isolation and Sequence Analysis of *OfCHS*

For cloning of CHS cDNA from *O. fragrans,* we aligned amino acid sequences of CHS from different plant species, and identified highly conserved regions of CHS from these

species. Following the conserved sequence, two degenerate primers DCHSF1 and DCHSR were synthesized to amplify the partial sequence of the CHS gene (Table 1). RT-PCR was performed with the degenerate primer pair. After an initial 98˚C for 2 min denaturation step, 35 cycles were run each with 10 s of denaturation at 98˚C, followed by 15 s annealing at 55˚C, and 2 min extension at 68˚C using MightyAmp® DNA Polymerase Ver.2 (TAKARA). The PCR product of about 1000 bp was cloned into pMD18-T vector (TaKaRa) and confirmed by sequencing.

The rapid amplification of cDNA end (RACE) approach was used to isolate the 3' and 5' ends of OfCHS cDNA by use of 3' SMARTer™ RACE cDNA Amplification Kit (Clontech) and 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 kits (Invitrogen). These primers for 5' RACE (A001-1, A001-2 and A001-3) and 3'RACE (3'F001-1 and 3'F001-2) were listed in Table 1. All reactions were performed according to the manufacturer's instructions. These amplified cDNA fragments were ligased into pMD18-T vector (TaKaRa) and sequenced. The CDS sequence of OfCHS and CHS sequences from other plant species was compared. These sequences were aligned by DNAMAN software and manually adjusted. Phylogenetic analysis was performed by use of DNAMAN version 6.0 (LynnonBio Soft company, USA).

2.3. Quantitative Real-Time PCR Analysis

The first strand cDNA was synthesized from 200 ng purified RNA using random hexamers at 37˚C for 60 min and RevertAid First Strand cDNA Synthesis Kit (THERMO SCIENTIFIC). For endogenous control, the 18sRNA probe was used. The real-time PCR was carried out with the *Power* SYBR® Green PCR Master Mix (Applied Biosystems) using theBIO-RAD CFX Connect™ instrument (Applied Biosystems). The PCR program included a 5 min denaturation step at 95˚C and then 45 cycles of 10 s of denaturation at 95˚C and 20 s of hybridization at 55˚C and 20 s of polymerization at

Table 1. Primers used to isolate and analyze the expression of *OfCHS* gene.

	Primer name	Primer sequence (5'-3')
Conserved area of the *OfDFR* gene	DCHSF1	GTBGAYCARAGCACBTAYCCNGA
	DCHSR1	CTRTGVAGCACMACVGTCTCVAC
5' RACE	A001-1	CCACTCCTTGATTGC
	A001-2	ACTTCCACCACCACAA
	A001-3	TCCGTCAAGTGCATGTAACG
3'RACE	3'F001-1	CCAAGTCGAAGCAACGTTAGCCCTA
	3'F001-2	TGAGATGAGGAAGTCCGCCACCAAA
RT-qPCR	QCHS-F	CAGCCTCACAAACTATACTACCAG
	QCHS-R	TCCCATACTCGCTTAGCACAT
	DCHSF1	GTBGAYCARAGCACBTAYCCNGA
	DCHSR1	CTRTGVAGCACMACVGTCTCVAC
Full-length cDNA	A001-1	CCACTCCTTGATTGC
	A001-2	ACTTCCACCACCACAA

72˚C. The relative expression ratios were calculated using the BIO-RAD CFX manage software vertion 3.1 sequence detection software (Applied Biosystems) and normalized using the 18S ribosomal RNA results. Real-time quantitative PCR was performed in three replicates for each sample. The primers used for *OfCHS*, and 18srRNA genes were listed in **Table 1**.

2.4. Analysis of Anthocyanin Relative Content

The ralative content and analysis of anthocyanin were carried out following the method described by Rabino and Mancinelli, 1986. Flower petals were obtained from different flowering stage of each cultivar. Three repetitions were performed. In brief, 0.3 g flower petals were ground in liquid nitrogen, and then extracted with appropriate solvent of 1% (hydrochloricacid: methanol) to 50 mL, immediately in 530, and 657 nm range scanning, and the total amount of anthocyanins ((A530-0.25A657)/g) was determined [12].

3. Results

3.1. Molecular Cloning and Sequence Analysis of *OfCHS*

Using RT-PCR and RACE approaches, we isolated OfCHS cDNA (GenBank accession number KR604813). The cDNA was 1383 bp long and a coding sequence (CDS) of 1173 bp encoding a polypeptide of 391 amino acids with an estimated molecular mass of 39.9 kDa. The theoreticalisoelectric point was 6.23. The protein sequences had three catalytic residues: Cys^{164}-His^{303}-Asn^{336}, and two Phe activeresidue (Phe^{215} and Phe^{265}) and the characteristics peptide sequence of chalcone synthasefamily (RFMMYQQGCFAGGTVLR and GVLFGFGPGL) were included [13] (**Figure 1(a)**). Amino acid sequence alignment of CHS from different plant species showed OfCHS shared 95% identity with the protein from *Oleaeuropaea*, and high similarity with those from other plants, such as *Rhododendron simsii* (89%), *Gossypium hirsutum* (79%), *Hibiscus cannabinus* (89%), *Malus domestica* (87%), *Sorbus aucuparia* (88%), *Camellia sinensis* (90%) (**Figure 1(b)**). The phylogenetic analysis demonstrated similar patterns, which indicated that CHSs from *Solenostemon scutellarioides*, *Perillaf rutescens*, *Antirrhinum majus*and *Digitalis lanatacan* be categorized into one group (**Figure 1(b)**). These results suggested that an evolutionary link did exist among these plants.

3.2. Expression of *OfCHS*

We analysed the expression pattern of *OfCHS* by Real-time quantification RT-PCR (qRT-PCR) approach. The expression pattern indicated that *OfCHS*s showed the highest transcript abundance in matureleaves, the lowest levels in flowers, which was very different toward several ornamental trees such as Tree Peony [14] (**Figure 2**). The expression of the initial flower stage (S2) was a little higher than that in full flowering stage (S3) (**Figure 2**). We also investigated the expression levels of *OfCHS* in different floral stage in two cultivars and showed that the transcripts in "Zi Yingui" petals were clearly higher than that in "Chenghong Dangui" at three stages especially at xiaoyan stage (S1) and there were no significant difference between the two cultivars in the full flowering stage (S3) (**Figure 3**).

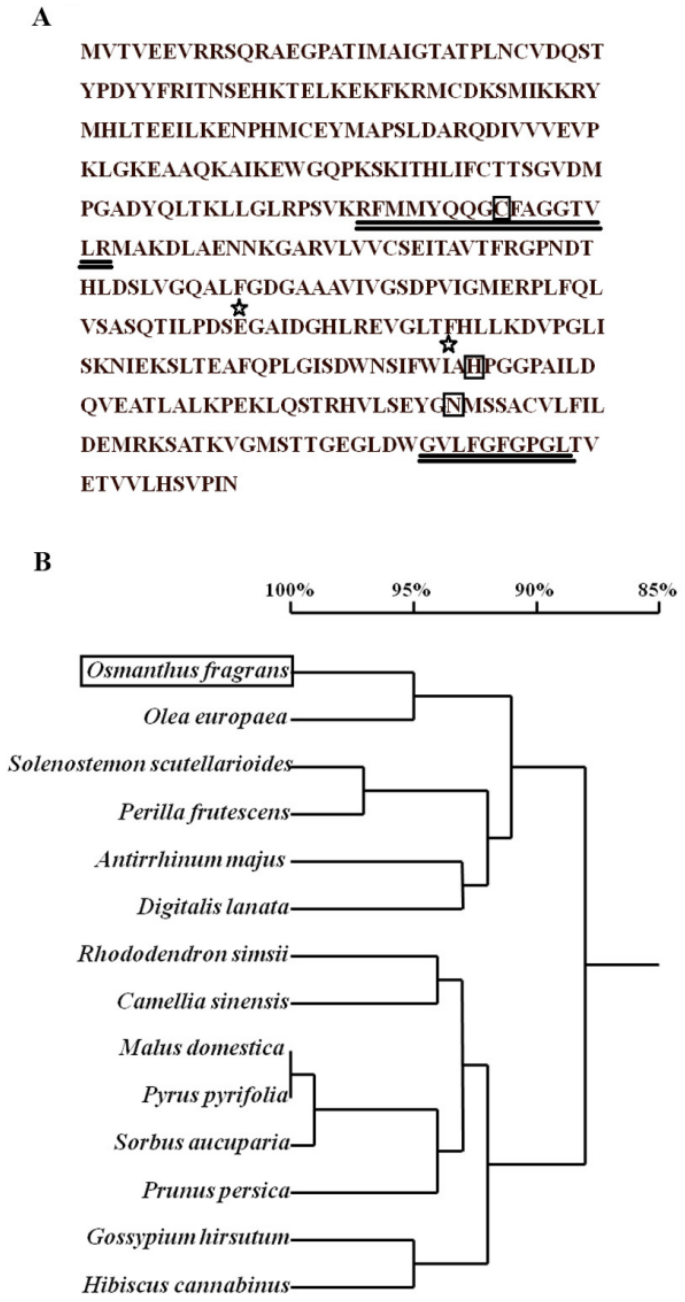

Figure 1. Sequence analysis of OfCHS. (a) Deduced amino acid sequences of OfCHS. The characteristics peptide sequence of chalcone synthasefamily (RLMMYQQGCFAGGTVLR and GVLFGFGPGL) are double-underlined. Two Phe active residue were marked by Asterisk Start. The catalytic triad sites C^{164}, H^{303} and N^{336} are boxed; (b) Phylogenetic analysis of OfCHS and other plant species. Accession number: *Olea europaea*, AHK07000.1; *Osmanthus fragrans*, KR604813; *Rhododendron simsii*, CAC88858.1; *Gossypium hirsutum*, ABS52573.1; *Hibiscus cannabinus*, AIC75908.1; *Sorbus aucuparia*, ABB89213.1; *Prunus persica*, XP_007223025.1; *Malus domestica*, XP_008380609.1; *Camellia sinensis*, P48387.1; *Pyrus pyrifolia*, AFQ92052.1; *Solenostemon scutellarioides*, ABP57071.1; *Perilla frutescens*, AA19548.1; *Antirrhinum majus*, P06515.1; *Digitalis lanata*, CAA05512.1; The tree is determined using the DNAMAN version 6.0.

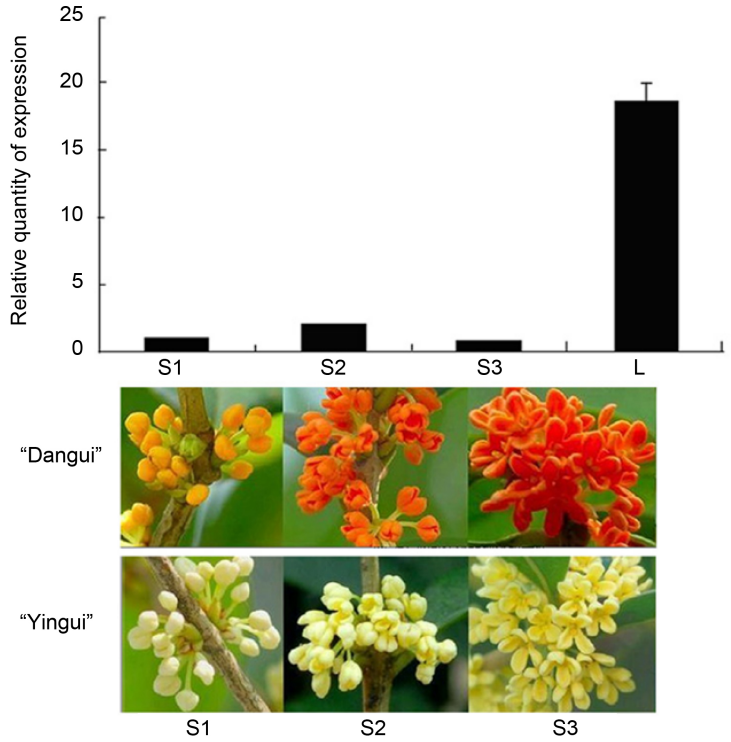

Figure 2. The relative expression of *OfCHS* gene in different flowering stages and leaves of *Osmanthus fragrans.* S1: xiangyan stage; S2: initial flowering stage; S3: full flowering stage; L: leaves.

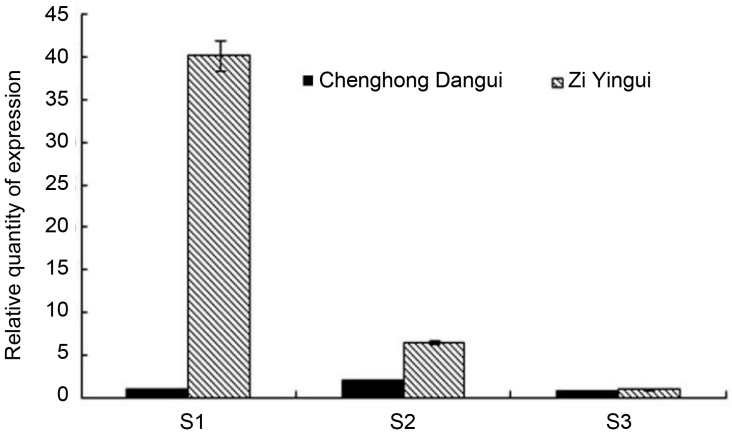

Figure 3. Relative quantity of *OfDFR* expression in different floral stages in "Chenghong Dangui" and "Zi Yingui". S1: xiangyan stage; S2: initial flowering stage; S3: full flowering stage.

3.3. Anthocyanin Content of Flower Petals

The results showed that total relative anthocyanin content was 0.74 $U \cdot g^{-1}$, 1.60 $U \cdot g^{-1}$ and 0.41 $U \cdot g^{-1}$, each at xiangyan stage, initial flowering stage and full flowering stage in "Dangui" flower petals and 0.34 $U \cdot g^{-1}$, 0.51 $U \cdot g^{-1}$, and 0.28 $U \cdot g^{-1}$ in "Yingui" flower petals (**Figure 4**). Statistical analysis showed that the anthocyanin content of "Dangui" was clearly higher than that in "Yingui" petals of three flowering stage (correlation is significant at the 0.05 level. $P < 0.05$).

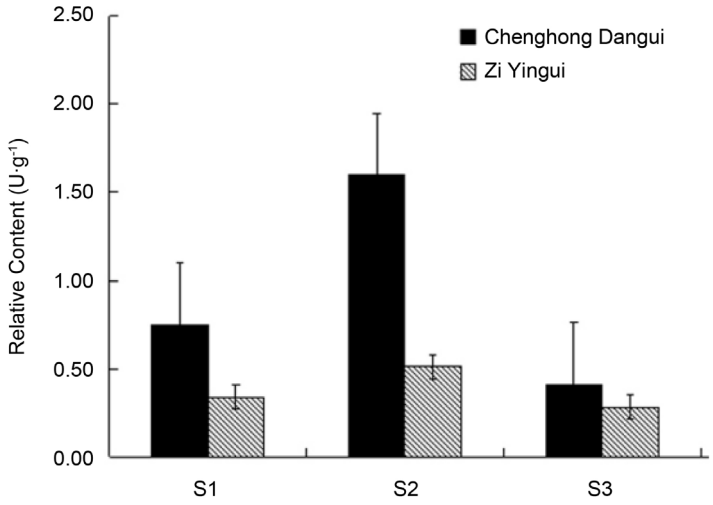

Figure 4. Relative contents of anthocyanin in the two cultivar petals. S1: xiangyan stage; S2: initial flowering stage; S3: full flowering stage.

4. Discussion

Chalcone synthases (CHSs) catalyze the condensation of p-coumaroyl-CoA and three malonyl-CoA molecules to form the naringenin chalcone, which is the first committed step in the flavonoids pathway of plants, leading to the biosynthesis of flavonoids, isoflavonoids, and anthocyanins [15]. Chalcone synthases have been extensively studied in higher plants especially in herbaceous plants.

In this study, a full length of cDNA encoding CHS protein of *O. frangrans* (*OfCHS*) was cloned by using RT-PCR and RACE PCR. This is the first time the gene and the expression of CHS in sweet osmanthus was analysed. The sequence analysis showed that the CHS has similar structure with that of higher plants.

The expression pattern indicated that *OfCHS* showed the highest transcript abundance in mature leaves, the lowest levels in S3 (**Figure 2**). The result was different from other ornamental plants which CHS expression was highest in petals. Moreover the transcripts in "Zi Yingui" petals were clearly higher than those in "Chenghong Dangui" at three stages especially at S1 and there was no significant difference between the two cultivars in S3, but the anthocyanin content of "Dangui" was clearly higher than that in "Yingui" petals of three flowering stage and this showed that the OfCHS did not result in the accumulation of total anthocyanin in "Dangui" (**Figure 3** & **Figure 4**).

Additional studies are needed to clarify the contribution of other enzymes or regulatory factors to the color formation in *O. frangrans.*

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