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Full Length Research Paper

Molecular characterization and expression analysis of chalcone synthase gene during flower development in tree peony (*Paeonia suffruticosa*)

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Chalcone synthase (CHS, EC: 2.3.1.74) is a key enzyme in the flavonoid and anthocyanin biosynthesis pathway. In order to investigate the role of CHS in tree peony flower coloration mechanism, we isolated and characterized the CHS gene from *Paeonia suffruticosa* cv. Yu Ji Yan Zhuang and analyzed its spatial and temporal expression patterns during floral development. The cDNA sequence of the CHS gene in *P. suffruticosa* (*Ps-CHS*1, genbank accession no. GQ483511) was 1475 bp in full length containing an opening reading frame (ORF) of 1185 bp that encoded a 394 amino acid polypeptide. Bioinformatic analysis showed that, Ps-CHS1 possessed all the conserved active sites for the CHS function as well as the family signature. Sequence alignment and phylogenetic analysis revealed that Ps-CHS1 shared high homology with CHS from plants in Salicaceae, Malvaceae and Rosaceae. The homology-based structural modeling showed that Ps-CHS1 had the typical structure of CHS. Southern blot analysis indicated that CHS was encoded by a small multigene family in the genome of tree peony. Anthocyanidin content in full-opening flower petals accumulated to the highest level. Real-time polymerase chain reaction amplification (PCR) analysis indicated that, *Ps-CHS*1 showed the highest transcript abundance in petals, moderate levels in sepals, low levels in leaves and stamens, and the lowest levels in carpels. *Ps-CHS*1 was actively expressed during flower development and increased gradually until reached maximal expression when flower fully opened. These results indicated that *Ps-CHS*1 was involved in the flower pigmentation of tree peony.

Key words: Tree peony (*Paeonia suffruticosa*), chalcone synthase, expression, anthocyanin.

INTRODUCTION

Flavonoids are ubiquitous natural plant products involved in insect pollination, UV protection, pigmentation, legume nodulation, disease and stress resistance (Winkel-Shirley, 2001a,b). Because of their biological and agricultural importance, flavonoid biosynthesis has been studied in flowers, fruits and kernels for many years. Using mutants or crossed lines of snapdragon, petunia, maize and *Arabidopsis* as model plants, nearly all enzymes involved in the flavonoid pathway have been identified and a large number of the structural genes as well as some regulatory genes have been isolated (Holton and Cornish, 1995; Tanaka et al., 2008).

Chalcone synthase (CHS; EC 2.3.1.74) is the first committed enzyme in flavonoid pathway and it catalyzes the synthesis of 2',4,4',6'-tetrahydroxy chalcone (THC) from one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA (Heller and Hahlbrock, 1980; Ferrer et al., 1999). THC provides the C6-C3-C6 skeleton of all

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Abbreviations: ORF, Opening reading frame; **CHS,** chalcone synthase; **THC,** 2',4,4',6'-tetrahydroxy chalcone; **CTAB,** cetyltrimethylammonium bromide; **cDNA,** complementary DNA; **DNase,** deoxyribonuclease; **RNase,** ribonuclease; **dNTP,** deoxyribonucleoside 5'-triphosphate; **RT,** reverse transcription; **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcriptase- PCR; **RACE,** rapid amplification of cDNA ends; **3D,** three-dimensional; **5'-UTR,** 5'-untranslation region; **3'-UTR,** 3'-untranslation region; **PKS,** polyketide synthase.

flavonoid compounds, it is then rapidly and stereo-specifically isomerized to yield the colorless (*2S*)-flavanones, which are the exclusive substrates for down-stream enzymes. Till date, various CHS mutants have been identified based on the flower or pollen phenotype from *Zea mays* (Franken et al., 1991), *Petunia hybrida* (Napoli et al., 1999) and others, and many *CHS* genes have been cloned from monocot, dicot and some gymno-sperm species such as *Z. mays* (Franken et al., 1991), *Bromheadia finlaysoniana* (Liew et al., 1998), *Arabidopsis* (Saslowsky et al., 2000), *Sorghum bicolor* (Lo et al., 2002) and *Ginkgo biloba* (Pang et al., 2005). All the cloned *CHS* genes are found to belong to a small multi-gene family. Furthermore, the spatial and/or temporal expression of *CHS* genes has been well characterized for *Z. mays* (Franken et al., 1991), *Gerbera hybrida* (Helariutta et al., 1995), *Ipomoea purpurea* (Durbin et al., 2000) and *Eustoma grandiflorum* (Noda et al., 2004). In support of the notion that CHS plays a critical role in flavonoid metabolism, successful reduction of antho-cyanin biosynthesis by down-regulating CHS through various gene silencing approaches has been reported in petunia (Krol et al., 1988), chrysanthemum (Courtney-Gutterson et al., 1994), rose (Gutterson, 1995), carnation (Gutterson, 1995), lisianthus (Deroles et al., 1998) and gentian (Nishihara et al., 2003).

Tree peony (*Paeonia suffruticosa*) is a very popular traditional ornamental plant in China and is also appreciated internationally because of its large showy flowers; cultivars with various flower colors have been produced by conventional breeding, especially in China. Analyses on compositions and amounts of petal pigments have been investigated in different groups and several wild species of Chinese tree peony (Wang et al., 2001, 2004; Zhang et al., 2007). The previous studies showed that major anthocyanins in tree peony were the 3-*O*-glucosides and 3, 5-di-*O*-glucosides of pelargonidin, cyanidin and peonidin, and major flavone and flavonol aglycones were apigenin, luteolin, kaempferol, quercetin, chrysoeriol and isorhamnetin (Hosoki et al., 1991; Wang et al., 2001; Wang et al., 2005).

Till now, although many genes and cDNA clones for the anthocyanin biosynthesis pathway have been isolated and well characterized for flowers of dicotyledon plants and for kernels of monocotyledon plants, little information, if any, is available concerning molecular aspect of flavonoid biosynthesis in tree peony. In this study, we isolated the full length cDNA clone encoding CHS from petals of tree peony, then, studied the predicted function of the enzyme encoded by this cDNA with homology research to known functional cDNA clones. We also presented its expression patterns in petals at different developmental stages and in different tissues to evaluate the relationship between the biosynthesis of anthocyanin and gene expression. Possible regulatory role of the *CHS* gene in tree peony flower coloration mechanism is discussed.

MATERIALS AND METHODS

Plant materials

Tree peony *P. suffruticosa* cv. Yu Ji Yan Zhuang (red flower cultivar) was grown in the Liangxiang peony base of Chinese Academy of Forestry (Beijing, China). Petal samples were collected at 6 different flower developmental stages (Figure 1a) for anthocyanin analysis and RNA extractions. Young leaves were obtained for genomic DNA extraction. Leaves, sepals, stamens and carpels were collected at full opening stage (Stage 6, Figure 1a) for RNA extractions. Samples were immediately frozen in liquid nitrogen and stored at –80°C until use.

Anthocyanin measurement

Anthocyanin analysis was performed according to the method of Meng and Wang (2004) with some modification. In brief, petal tissues at each developmental stage were ground in liquid nitrogen and anthocyanin was extracted with 1% HCl/methanol for 24 h at 4°C. After clearing the extractions by centrifugation at 12,000×g for 30 min, the supernatant was analyzed with a Beckman DU-800 spectrophotometer (Beckman Instruments, Fullerton, CA). A_{530} minus $1/4A_{657}$ was used as a measure of the anthocyanin content; values were normalized to the fresh weight of each sample.

Isolation and sequencing of the full-length cDNA

Total RNA was isolated by the modified cetyltrimethylammonium bromide (CTAB) method (Chang et al., 1993) from fully opened flower petals. First-stand cDNA synthesis was performed using M-MuLV reverse transcriptase (Promega, USA). In order to obtain the *CHS* homologue from tree peony, degenerated oligonucleotide set of 5'-CA(A/G)CCCAAGTCCAA(A/G)AT(C/T) ACCC-3' (forward) and 5'-(A/T)CCCCACTC(A/C/G)AG(C/T/G)CCTTC(A/T) CC-3' (reverse), which were designed according to the conserved sequences of previously cloned *CHS* genes, were used. PCR reactions were carried out for 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C, 40 s at 72°C and final elongation for 7 min at 72°C. The amplified RT-PCR products were analyzed on a 1.2% agarose gel and specific band of expected size was purified by PCR purification kit (Tiangen, China). Isolated DNA fragment was TA-cloned into the pGEM-T easy vector (Promega, USA) and then transformed into competent Top10 *Escherichia coli* cells for sequencing.

To determine the full-length nucleotide sequence, RACE-PCR was performed according to the user manual of SMART™ Race cDNA amplification kit (Clontech, Japan). The 5'-end fragment was amplified using specific primer GSP1 (5'-CACCGGAGGTGGTAC AGAAAACAAGGTG-3'), and the 3'-end fragment was amplified using specific primer GSP2 (5'-GGAAGAGGTCACTTGAGGAA GGAAAGGC-3'). Each primer was designed according to the nucleotide sequence of the cDNA fragment obtained from the RT-PCR. The products were cloned into the pGEM-T easy vector (Promega, USA) and then sequenced.

After comparing and aligning the sequence of 5' RACE, 3' RACE and the internal region products, the full-length cDNA sequence was obtained through PCR amplification using 3'-Ready cDNA as the template and a pair of specific primers P1 (5'-ATGGC TTCGGTTGAAGAAATTAG-3') and P2 (5'-TCACTCACTGATTG TAATTGCAGG-3') under the following condition: 94°C for 4 min, followed by 32 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 1 min 30 s) and final elongation for 7 min at 72°C. Cloning and sequencing of the full-length cDNA were performed using the methods described earlier.

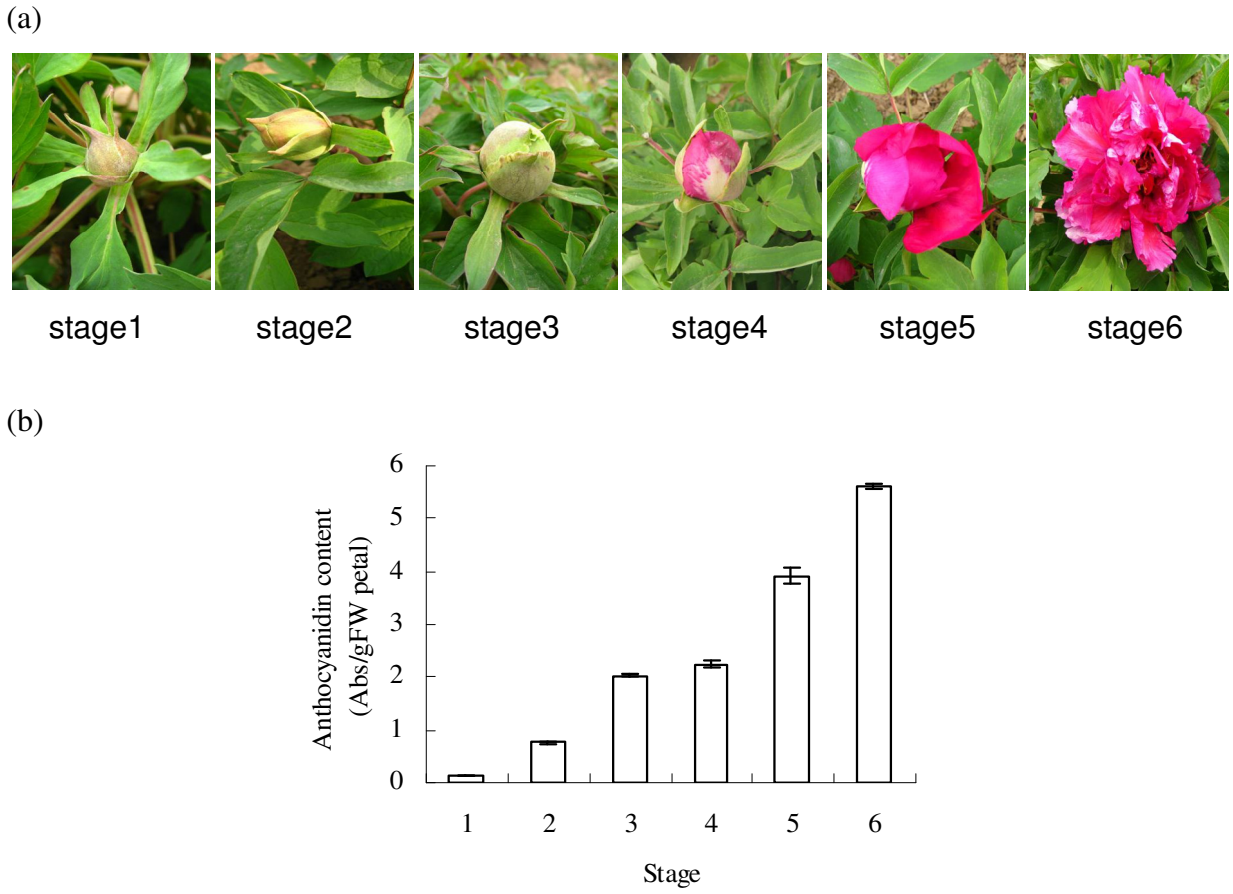

Figure 1. Developmental stages of the flower and accumulation of anthocyanin at each developmental stage of petals in tree peony cv. Yu Ji Yan Zhuang. (a) Tree peony flower developmental stages. stage 1, unpigmented tight bud; stage 2, slightly pigmented soft bud; stage 3, slightly pigmented bud just before anthesis; stage 4, initially opened flower; stage 5, half opened flower; stage 6, fully opened flower with exposed anthers. (b) Changes in anthocyanin accumulation at six developmental stages of petals. 1 to 6, Floral developmental stages. Vertical bars indicate standard error of three replicates.

Bioinformatic analyses

Sequence assembling was carried out with DNAStar. Comparative and bioinformatics analyses of the nucleotide sequences, deduced amino acid sequences and ORF were performed online at NCBI (http://www.ncbi.nlm.nih/gov) and ExPASy (http://expasy.org/tools/dna.html), respectively. Three-dimensional (3D) structure prediction of the deduced protein was performed by Swiss model workspace (http://swissmodel.expasy.org/). The multiple alignments and phylogenetic analysis based on putative complete amino acid sequence were dealt with DNAMAN ver. 6.0.3.99 (Lynnon Biosoft).

Southern blot analysis

Genomic DNA was isolated from tree peony young leaves by the CTAB method (Murray and Thompson, 1980). Aliquots of genomic DNA (30 µg) were digested overnight at 37℃ with appropriate restriction endonucleases, *Eco*RI, *Eco*RV and *Bam*HI (Takara, Japan), respectively, separated by electrophoresis on a 0.8% (w/v) agarose gel in TAE buffer and transferred onto a positively charged Hybond-N^+ nylon membrane (Amersham Biosciences, UK). An aliquot of 50 ng purified coding sequence of the full-length cDNA was used as a template in a total volume of 20 µl for probe labeling. Probe labeling, hybridization and signal detection were performed according to the manufacturer's protocol of DIG high primer DNA labeling and detection starter kit II (Roche, Germany). Experiment was repeated at least twice.

Relative-quantitative real-time PCR

Total RNA samples were prepared from petals at different developmental stages and different tissues of tree peony (leaves, sepals, stamens and carpels). After treated with RNase-free DNase I (Tiangen, China) according to the user manual, 1 µg of total RNA was reverse-transcribed to the first-stand cDNA using the PrimeScript® RT reagent kit (Takara, Japan). Relative-quantitative real-time PCR reactions were performed in a 96 well plate with an ABI Prism 7500 sequence detector (Applied Biosystems, USA), using SYBR® premix Ex Taq™ Kit (Takara, Japan) to monitor cDNA amplification, according to the manufacturer's protocol. As a control, parallel amplification reactions of the tree peony house-keeping gene *beta-Tubulin* (GenBank no. EF608942) were also performed. Each primer set was designed based on the 3'-end cDNA sequence of the corresponding gene. The specific primers

used for real-time PCR were as follows: for Ps-*CHS*1, 5'-AGCAGAGAACAACAAAGGGTCACG-3' (Forward) and 5'-TCAGCACCGA CAATAACCGCAG-3' (Reverse), giving a product of 270 bp; for *beta-Tubulin*, 5'-TGAGCACCAAAGAAGTGGACGAAC-3' (Forward) and 5'-CACACGCCTGAACATCTCCTGAA-3' (Reverse), giving a product of 182 bp. The reaction mix (20 µl) contained 2 µl RT-product, 0.4 µl (10 µM) for each forward and reverse primers, 10 µl SYBR® Premix Ex Taq™ (2×) and 0.4 µl ROX reference dye II. Thermal cycling conditions were: 95°C for 30 s and 40 cycles of 95°C for 5 s, 60°C for 34 s; then 95°C for 15 s, 60°C for 20 s and 95°C for 15 s for the dissociation stage. After the real-time PCR, the absence of unwanted by-products was confirmed by automated melting curve analysis and agarose gel electrophoresis of the PCR product. The amplified DNA fragments (270 bp) were sequenced to confirm the amplified fragment codes for a partial *Ps-CHS*1 cDNA.

In all experiments, five replicates for each RNA sample were included; averages were calculated and differences in the threshold cycle (Ct) were evaluated by 7500 System Sequence Detection Software v1.3.1. For data analysis, the comparative Ct method (ABI Prism 7700 Sequence Detection System User Bulletin #2, Applied Biosystems, USA) was used, which mathematically transforms the Ct data into the relative transcription level of genes. When comparing the expression of *Ps-CHS*1 in different tissues, the relative quantification of the *Ps-CHS*1 expression was achieved by calibrating its transcription level to that of the reference gene, *beta-Tubulin*. When analyzing the expression of *Ps-CHS*1 in petals of different developmental stages, the transcription level of *Ps-CHS*1 in petals of stage 1 was used as the calibrator and defined as one. The expression level calculated by the formula $2^{-\Delta\Delta Ct}$ represents the x-fold difference from the calibrator.

RESULTS

Isolation and characterization of cDNA encoding CHS

Using degenerated primers derived from conserved sequences of previously cloned *CHS* genes, the study first amplified a partial cDNA fragment by RT-PCR. GenBank Blastn search analysis indicated that, the cDNA fragment with 751 bp in length showed high homology to known *CHS* sequences from other plant species. Subsequently, a fragment of approximately 500 bp at 5'-end and a fragment of approximately 350 bp at 3'-end was amplified by 5'/3' RACE, respectively. Finally, the full-length cDNA sequence was obtained by sequences assembling and ORF was amplified through RT-PCR using the specific primers. Nucleic acid sequence alignment of the full-length cDNA revealed high levels of sequence similarity to other *CHS* genes. Thus, the study considered this full length cDNA as the cDNA of *CHS* and named it *Ps-CHS*1 (GenBank accession No. GQ483511).

DNA sequencing revealed that, *Ps-CHS*1 was 1475 bp in full length and contained a 5'-untranslation region (5'-UTR) of 82 bp, a 3'-untranslation region (3'-UTR) of 208 bp with a poly (A) tail and an ORF of 1185 bp encoding a polypeptide of 394 amino acids (Figure 2). The deduced Ps-CHS1 protein had a predicted molecular weight of 43.3 kDa and a pI of 6.19. Further sequence analysis of the putative amino acids indicated that, Ps-CHS1 contained the active sites for the CHS function "RLMMYQQGCFAGGTVLR" (156 to 172) as well as the family signature "GVLFGFGPGL" (368 to 377) (Lanz et al., 1991; Helariutta et al., 1995; Ferrer et al., 1999; Kim et al., 2002). Moreover, Ps-CHS1 contained the active amino acid residues highly conserved among all CHS sequences characterized thus far, including seven amino acid residues of the cyclization pocket, three catalytic triad sites, five residues of coumaroyl pocket and three CoA binding active sites. The most significant active-site amino acid residues responsible for the reaction of multiple decarboxylation and condensation were identified as Cys164, Phe215, His303 and Asn336, which were also conserved in Ps-CHS1 (Schröder et al., 1998; Ferrer et al., 1999) (Figure 2).

Multiple alignments and phylogenetic analysis of Ps-CHS1

Alignments of the deduced amino acid sequences showed that the protein of *Ps-CHS*1 shared high degree of identity (86-91%) with CHS sequences isolated from various plant species, such as *Populus alba*, *Citrus sinensis*, *Camellia sinensis*, *Rosa hybrid*, *Malus* × *domestica and Glycine max,* which suggests that *Ps-CHS*1 belongs to the CHS family (Table 1).

The homology-based 3D structural modeling of Ps-CHS1 was analyzed by Swiss-Modeling using the crystal structure of CHS from alfalfa (Ferrer et al., 1999) as template. 3D structure of Ps-CHS1 (Figure 3) shared 82.86% similarity with the template, which further facilitated positive identification of its CHS identity.

Phylogenetic analysis derived from a number of CHS protein sequences including Ps-CHS1 showed that they were grouped into two distinct clades; CHS proteins from dicotyledons constituted a monophyletic group, while those from monocotyledons were clustered into another distinct clade (Figure 4). This result was similar to the previous research (Nakatsuka et al., 2003). Ps-CHS1 was located in the cluster of dicotyledon CHSs which was further divided into several subgroups based on the different plant species, for example, CHS proteins from *Abelmoschus manihot* and *Gossypium hirsutum* (both in the Malvaceae family) appeared in the same subgroup and those from *R. hybrid* and *malus* × *domestica* (both in the Rosaceae family) were nested in another subgroup (Figure 4). These results suggest that CHS is well-conserved among plants of different groups and has distinct species specificity.

Southern blot analysis

To examine the copy number of the *Ps-CHS*1 gene in *P. suffruticosa* cv. Yu Ji Yan Zhuang, aliquots of 30 µg genomic DNA were digested with *Eco*RI, *Eco*RV and *Bam*HI, respectively, which did not cut within the coding

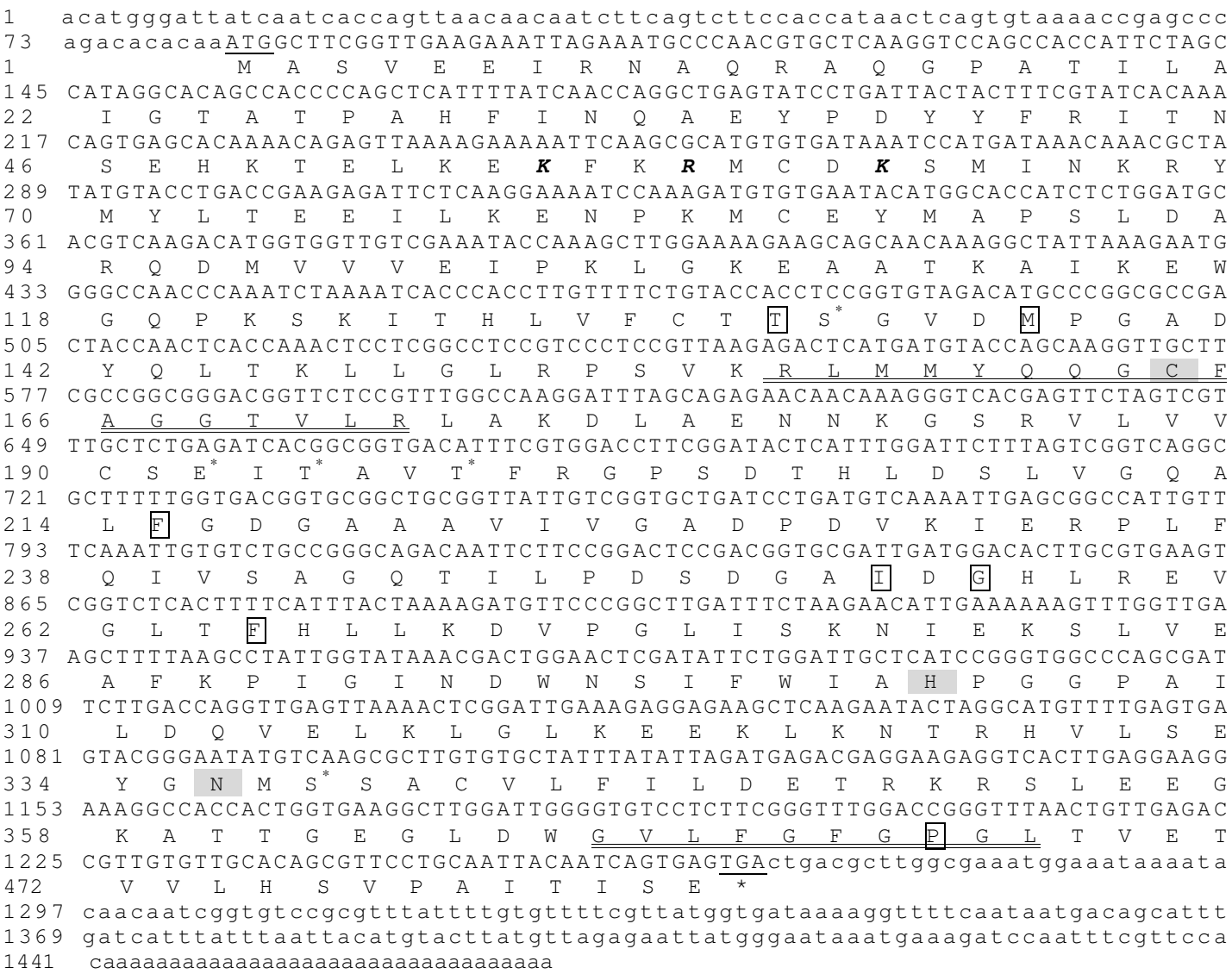

Figure 2. Nucleotide and deduced amino acid sequences of the full-length cDNA of *Ps-CHS*1. The small letters were untranslated sequence and the capital letters were coding sequence. The initiation (ATG) and termination codons (TGA) were underlined. The seven amino acid residues of the cyclization pocket, including the sites of Thr132, Met137, Phe215, Ile254, Gly256, Phe265 and Pro375 were framed; the catalytic triad sites Cys164, His303 and Asn336 were shaded, while * denoted the residues of coumaroyl pocket, including Ser133, Glu192, Thr194, Thr197 and Ser338. The family signatures of chalcone synthase (RLMMYQQGCFAGGTVLR and GVLFGFGPGL) were double-underlined. The CoA binding active sites such as Lys55, Arg58 and Lys62 were italic and bold.

sequence region and then, hybridized with the coding sequence of *Ps-CHS*1 under high stringency condition. The results revealed that there were several specific hybridization bands ranging from 23.0 to 1.0 kb in each lane, indicating that *Ps-CHS*1 belonged to a small multi-gene family in tree peony (Figure 5).

Accumulation of anthocyanins during floral development

The accumulated amounts of anthocyanins in petals were measured temporally throughout the floral development (Figure 1b). From small buds to pigmented flowers, 6 stages were classified as described in materials and methods. In tree peony cv. Yu Ji Yan Zhuang, flower pigmentation was not observed at early bud stage (Stage 1), but spots at the basal part of petals appeared and started to accumulate anthocyanins at this stage. As shown in Figure 1b, anthocyanins accumulation was at a very low level in small buds (Stage 1) and thereafter increased sharply from stage 1 to 6. The anthocyanins concentration reached the maximum when flower fully opened (Stage 6) with a level of more than 40 times higher than

Table 1. Percent similarity and identity of Ps-CHS1 amino acid sequence with CHS genes from other plant species.

Plant species	Accession no.	Sequence identity (%)	Sequence similarity (%)
P. suffruticosa cv. Yu Ji Yan Zhuang	(GQ483511)	100	100
P. alba	(ABD24222)	91	96
C. sinensis	(ACB47461)	90	94
C.sinensis	(P48386)	88	95
R. hybrid	(BAC66467)	87	93
Malus × *domestica*	(AAX16492)	87	94
G. max	(CAA46590)	86	93

no. means number.

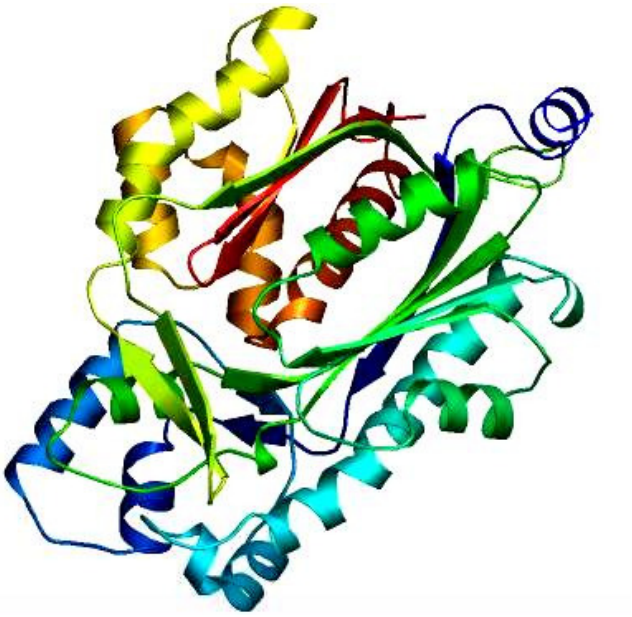

Figure 3. The computational 3D structure of Ps-CHS1.

that at stage 1 (Figure 1b).

Expression profiles of *Ps-CHS*1 in different floral developmental stages and multi-tissues

To investigate the tissue-specific and developmental expression patterns of *Ps-CHS*1, relative-quantitative real-time PCR with gene-specific primers was performed to detect expression levels of this transcript. cDNA prepared from leaves, sepals, stamens and carpels collected at full opening stage (Stage 6), and petals at each floral developmental stage were used as templates, respectively. The primers were positioned at variable regions to avoid the possible amplification of other CHS genes. The results showed that *Ps-CHS*1 transcript accumulated in petals, leaves, sepals, stamens and carpels, but the relative expression levels varied significantly. *Ps-CHS*1 showed the highest transcript abundance in petals, moderate levels in sepals, low levels in leaves and stamens and the lowest levels in carpels (Figure 6).

Expression analysis of *Ps-CHS*1 in petals during flower development showed that, *Ps-CHS*1 was actively expressed throughout floral development; *Ps-CHS*1 transcript increased gradually from stage 1 to 5 and reached maximal level at stage 6, which was temporally related to anthocyanins accumulation (Figure 7). These results revealed that *Ps-CHS*1 expression was tissue-specific and developmentally regulated in tree peony.

DISCUSSION

In the present study, we isolated a cDNA clone encoding *CHS* homologue from petals of tree peony cv. Yu Ji Yan Zhuang, named *Ps-CHS*1 and characterized its spatial and temporal expression patterns during flower development.

CHS is the well-known representative of the type III polyketide synthase (PKS) super family. Previous studies have shown that type III PKSs from plant origin have approximately 400 amino acid long polypeptide chains (41-44 kDa) and share from 46 to 95% sequence identity (Flores-Sanchez and Verpoorte, 2009). Sequence analysis and comparison of the novel tree peony *Ps-CHS*1 revealed that the ORF was 1185 bp in length and putatively encoded a polypeptide of 394 amino acids, which had high similarities (86 to 91%) with CHSs from other plant species (Table 1), with a predicted molecular mass of 43.3 kDa. Ferrer et al. (1999) studied the detailed active-site architecture of CHS by analyzing the crystal structure of CHS2 isolated from alfalfa. The structure reveals that four chemically reactive residues (Cys164, Phe215, His303 and Asn336), which are conserved in all the known PKSs (Flores-Sanchez and Verpoorte, 2009), define the active site and that five residues (Ser133, Glu192, Thr194, Thr197 and Ser338)

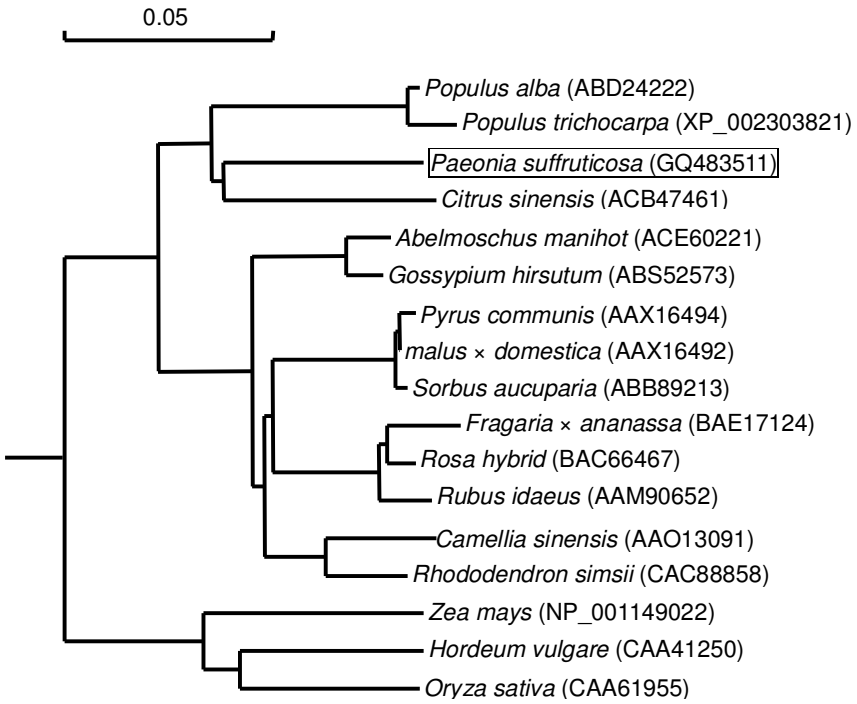

Figure 4. A phylogenetic tree based on the deduced amino acid sequences of various CHSs. The Ps-CHS1 protein is boxed.

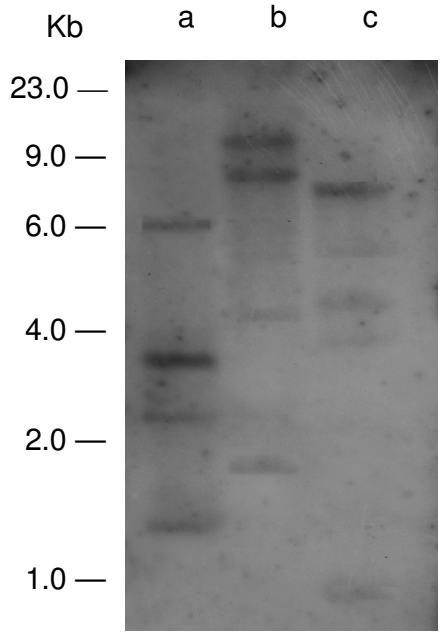

Figure 5. Southern blot analysis of *Ps-CHS*1 gene. 30 µg of tree peony genomic DNA was digested with *Eco*RI (lane a), *Eco*RV (lane b) and *Bam*HI (lane c), separated on a 0.8% agarose gel. The gel was blotted onto nylon and hybridized to the DIG-labeled probe of *Ps-CHS*1 cDNA. The estimated sizes for DNA bands are indicated in the left margin.

form the coumaroyl-binding pocket, while seven residues (Thr132, Met137, Phe215, Ile254, Gly256, Phe265 and Pro375) form the cyclization pocket. Further sequence analysis of the putative amino acids indicated that, Ps-CHS1 contained the family signature as well as all the active amino acid residues highly conserved among all CHS sequences (Figure 2). Moreover, the results of 3D structural modeling and phylogenetic analysis demonstrated its CHS identity (Figures 3 and 4). These findings strongly suggest that the novel tree peony *Ps-CHS*1 characterized in this study is a homologue of the *CHS* gene and protein of it is a typical CHS protein.

Southern blot analysis under high stringency detected multiple hybridizing bands indicating the possibility of other genes encoding CHS or pseudogenes were not identified in the present study (Figure 5). CHS was shown to be represented by multigene family in most plants studied, with different members of the family responding not only to various environmental stimuli such as wounding, UV irradiation and pathogen infecting, but also developmentally and tissue-specifically. To determine the spatial expression of *Ps-CHS*1 in tree peony, relative-quantitative real-time PCR analysis was carried out with total RNA extracted from 5 different tissues and organs. The expression analysis revealed that when compared with carpels, leaves and stamens, *Ps-CHS*1 was preferentially expressed in petals and sepals, the tissues which both have the potential to accumulate anthocyanins in different tree peony varieties (Figure 6). Meanwhile, the expression of *Ps-CHS*1 was particularly strong in

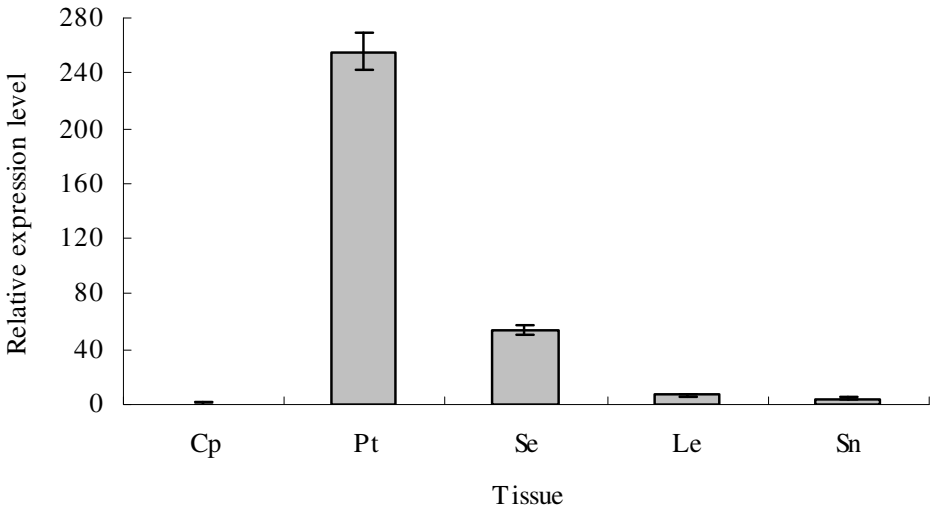

Figure 6. Expression profiles of *Ps-CHS*1 in different tissues collected at full opening stage. Real-time PCR analyses were performed using total RNA from carpels (Cp), petals (Pt), sepals (Se), leaves (Le) and stamens (Sn). *Ps-Tubulin* was used as an internal control. The expression of *Ps-CHS*1 in Cp was used as a calibration standard.

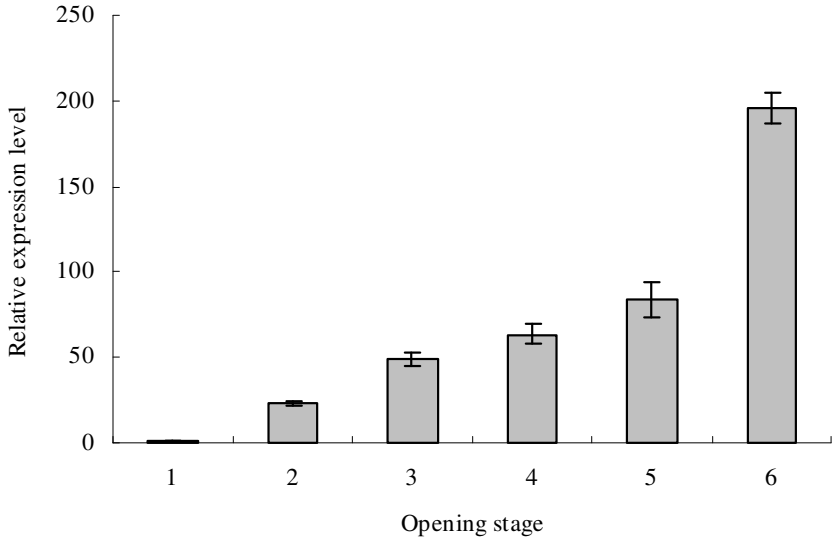

Figure 7. Expression profiles of *Ps-CHS*1 in petals at different floral developmental stages. Real-time PCR analyses were performed using total RNA from petals at each floral developmental stage (1 to 6). *Ps-Tubulin* was used as an internal control. The expression of *Ps-CHS*1 in petals at stage 1 was used as a calibration standard.

anthocyanin-pigmented petals (Figure 6), suggesting that *Ps-CHS*1 is spatially responsible for anthocyanin biosynthesis. As for *CHS* transcripts detected in organs lacking anthocyanin, it might be due to the expression of *CHS* gene involved in the biosynthesis of other secondary metabolites, such as flavones and flavonols. Similar results were also reported in *G. hybrida* (Helariutta et al., 1995), Asiatic hybrid lily (Nakatsuka et al., 2003), and *Dendrobium* orchid (Mudalige-Jayawickrama et al., 2005).

Subsequently, in order to elucidate the relationship between the *Ps-CHS*1 expression and anthocyanin accumulation, 6 stages were classified from small buds to pigmented flowers and the temporal expression pattern during floral development was determined. As shown in Figures 1b and 7, the expression of *Ps-CHS*1 increased

as the flower developed and reached the maximum level at stage 6; the expression pattern paralleled the increase in anthocyanin pigmentation in petals. These results indicated that *Ps-CHS*1 might play an important role during flower pigmentation in tree peony and the activity of CHS enzyme is regulated at a transcriptional level. The spatial and/or developmental expression of CHS gene, which is accompanied by anthocyanin biosynthesis, is also observed in other plant species. In lisianthus cv. Asuka no Sora (purple flower), *CHS* was most strongly expressed in petals and sepals and two peaks were observed in its expression patterns, which corresponded to the stage of flavonol biosynthesis and anthocyanin biosynthesis, respectively (Noda et al., 2004). In Chinese cabbage-pak-choi, *BcCHS* expressed at high levels in anthers and petals in later flower developmental stages and the transcripts were not detected in stages I, II, III flower buds, stems, sepals, filaments, pistils and leaves (Jiang and Cao, 2008).

Conclusion

In this study, we isolated and characterized a *Ps-CHS*1 cDNA clone in tree peony and by expression analyses, proposed that *Ps-CHS*1 was involved in the flower pigmentation. To our knowledge, this is the first report dealing with the mechanism of tree peony flower pigmentation at the molecular level. The cloning and expression analysis of other genes related to flavonoid accumulation in tree peony may pave the way to elucidate the molecular basis of its flower pigmentation and can also facilitate to the development of new cultivars of tree peony with different colors by manipulating flavonoid structural and regulatory genes through biotechnology techniques.

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