RESEARCH ARTICLE

Chalcone synthase genes from milk thistle (*Silybum marianum*): isolation and expression analysis

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Abstract

Silymarin is a flavonoid compound derived from milk thistle (*Silybum marianum*) seeds which has several pharmacological applications. Chalcone synthase (CHS) is a key enzyme in the biosynthesis of flavonoids; thereby, the identification of *CHS* encoding genes in milk thistle plant can be of great importance. In the current research, fragments of *CHS* genes were amplified using degenerate primers based on the conserved parts of Asteraceae *CHS* genes, and then cloned and sequenced. Analysis of the resultant nucleotide and deduced amino acid sequences led to the identification of two different members of *CHS* gene family, *SmCHS1* and *SmCHS2*. Third member, full-length cDNA (*SmCHS3*) was isolated by rapid amplification of cDNA ends (RACE), whose open reading frame contained 1239 bp including exon 1 (190 bp) and exon 2 (1049 bp), encoding 63 and 349 amino acids, respectively. *In silico* analysis of SmCHS3 sequence contains all the conserved CHS sites and shares high homology with CHS proteins from other plants. Real-time PCR analysis indicated that *SmCHS1* and *SmCHS3* had the highest transcript level in petals in the early flowering stage and in the stem of five upper leaves, followed by five upper leaves in the mid-flowering stage which are most probably involved in anthocyanin and silymarin biosynthesis.

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Introduction

Milk thistle, a member of the Asteraceae family is a known medicinal plant (annual/biennial). Having originated in the Mediterranean Basin, it is now widespread throughout the world (Morazzoni 1995; Kren and Walterova 2005). Seeds and fruits of milk thistle contain silymarin, a constitutive natural compound, which is an isomeric mixture of the silybin A, silybin B, isosilybin A, isosilybin B, silydianin, flavonolignans silychristin, isosilychristin and a flavonoid taxifolin (Morazzoni and Bombardelli 1995; Lee and Liu 2003; Davis-Searles *et al.* 2005). The seeds contain the highest amount of silymarin but the whole plant is used for medicinal purposes, and extracts of the fruit are used for the therapy of liver diseases including cirrhosis (Flora *et al.* 1998). There is a growing interest inits anticancer and chemopreventive effects as well as cardioprotective, neuroactive, hypocholesterolemic and neuroprotective activities (Kren and Walterova 2005).

Chalcone synthase (CHS) is a key enzyme for the biosynthesis of flavonoid antimicrobial phytoalexins and anthocyanin pigments in plants. It catalyzes the condensation of 4-hydroxycin-namoyl-CoA and three malonyl-CoA molecules to produce the chalcone derivative, naringenin chalcone, which is the first committed step in the phenylpropanoid pathway of plants (Ferrer *et al*. 1999). Due to the specialized function of different kinds of flavonoids, the activity of *CHS* is particularly regulated by endogenous mechanisms in plant development and tissue differentiation such as flower development (Liew *et al.* 1998; Van der Meer *et al.* 1992; Claudot *et al.* 1999; Pang *et al.* 2005) or by various external stimuli, such as low temperature (Leyva *et al.* 1995), UV treatments and blue light (Hartmann *et al.* 1998; Wade *et al.* 2001; Zhou *et al.* 2007), elicitor treatments such as salicylic acid and

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methyl jasmonate (Schenk *et al.* 2000), wounding (Richard *et al.* 2000), and pathogen attack (Soylu 2006).

In accordance with their important roles in initiating flavonoid biosynthesis, *CHS* genes have been cloned and characterized in many plant species. Most of the enzymatic components of plant secondary metabolism are encoded by small gene families, originated through gene duplications (Durbin *et al.* 2000). CHS is also encoded by a small multigene family in many plants (Martin 1993).

In this study, cloning and characterization of a full-length *CHS* cDNA and two partial genes from milk thistle are reported. Also, its expression patterns are presented in different tissues in different developmental stages to evaluate the relationship between the biosynthesis of silymarin and gene expression pattern.

Material and methods

Plant materials and growing conditions

Milk thistle (*S. marianum,* Majar genotype) seeds were obtained from Institute of Medicinal Plants (ACECR, Iran). Surface of the seeds were sterilized by 70% (v/v) ethanol for 3 min, 20% (v/v) sodium hypochlorite, and 0/1% (v/v) tween 20 for 15 min. Then, they were rinsed with sterile water thrice for 5 min. The seeds were cultured on MS medium supplemented with 1% agar according to the manufacturer's instruction (Duchefa Biochemie, Netherlands), and incubated in the growth chamber under the photoperiod/temperature cycle of 16 h light/25°C and 8 h darkness/20°C up to the four leaf stage. Then, the plants were cultured in pots containing an appropriate soil mixture and left to grow in the ABRII greenhouse at 27 ± 2°C.

Multiple sequence alignment and design of CHS degenerate primers

The available sequences of *CHS* genes from different plants were collected from National Center for Biotechnology Information (NCBI) and aligned using Clustal W (Larkin *et al*. 2007) to detect the conserved and diverged regions. Then, 4 degenerate primers including two forward (F1 and F2) and two reverse (R1 and R2) primers were designed based on the CHS consensus sequences in Asteraceae (figure 1, table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/) and synthesized in the MWG Company (Ebersberg, Germany).

DNA extraction and cloning of the genomic fragment

Genomic DNA was extracted using the Core-One^{TM} Plant Genomic DNA Isolation kit (CoreBio, Korea) according to its instructions. Polymerase chain reaction (PCR) was carried out using a termocycler Gradient 96 (Biorad, Berkely, USA) and conditions were optimized for F1R1, F1R2, F2R1 and F2R2 primer pairs. The PCR cycling schemes were: 5 min at 95°C, 40 cycles of 1 min at 95°C, 1 min at 55–64°C (depending on annealing temperature of the primer pairs), 1–2 min at 72°C, and 5–15 min final extension at 72°C. PCR reactions were performed in eppendorf tubes, each containing 0.5 μL of each primer (10 μM), 0.6 μL of 10 mM dNTPs, 2.5 μL of 10x PCR Buffer (Fermentas, Hanover, Germany), 0.2 μL of 5 U/μL *Taq* polymerase (Fermentas), 2.5 μL of 25 mM $MgCl_2$, and 100-ng DNA. Also, sterile distilled water was added to reach the final volume of 25 μL. Fragments of CHS genes amplified from milk thistle were detected by gel electrophoresis followed by staining with ethidium bromide PCR products were purified from an agarose gel using the DNA extraction kit (Fermentas), cloned into the pGEM-T easy vector (Promega, Madison, USA) according to the manufacturer's instruction and sequenced (Macrogen, Korea).

Design of specific primers

Six specific primers (three forward and three reverse) were designed based on the diverged regions of F1R2 (S*mCHS1*) and F2R1 (*SmCHS2*) fragments for the amplification of the related cDNA from milk thistle's total RNA in the RACE (rapid amplification of cDNA ends) system using Oligo primer analysis software (ver. 5.0; National Bioscience Inc., Plymouth, USA) (see table 1 in electronic supplementary material). Moreover, two primer pairs were designed for real-time PCR to evaluate the expression of *SmCHSs* and a specific primer pair was specified for 18s rRNA as the

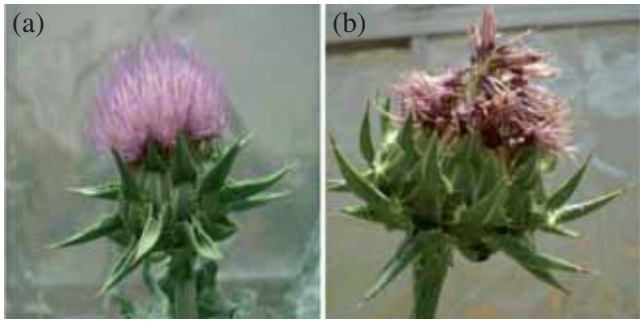
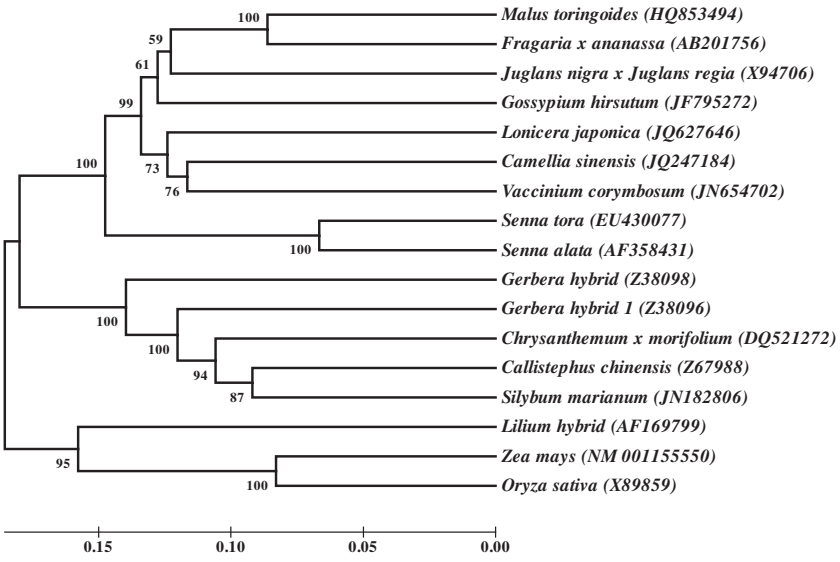


Figure 1. Two developmental stages of milk thistle (*S. marianum*): (a) early flowering; (b) mid-flowering.



(a)

(b)

Figure 2. (A) Phylogenetic analysis of *C. synthase* cDNA sequence of *Silybum marianum* (*CHS3*) along with *CHS* sequences of different plant species using the unweighted pair group method using arithmetic mean (UPGMA) clustering procedure. (B) Alignment of the CHS amino acid sequence fragments from some Asteraceae family species. The sequences around the active site residues, Cys, Phe, His and Asn and the conserved CHS signature (GFGPG loop) are shown.

internal control (table 1 in electronic supplementary material). It is noteworthy that due to the high sequence similarity of *SmCHS1* and *SmCHS3* (98%), *CHS1*F and *CHS1*R primer pairs could also amplify *SmCHS3* (figure 2 in electronic supplementary material), and design of suitable specific primers which exclusively amplify one of them was not possible.

RNA isolation and cloning of RACE fragments

Total RNA was isolated from the petals of milk thistle using Trizol reagent (Life Technology, Invitrogen, Foster City, USA) according to the manufacturer's instruction. DNA was removed by treating with RNase-free DNase I (Promega, Madison, USA). To determine the full-length nucleotide sequence of each *CHS*, rapid amplification of cDNA ends (both 3′ and 5′) were performed using RACE system (Invitrogen) according to the supplier's instruction. The 3′-end fragments were amplified using specific forward primer 2 (CHS1F2 and CHS2F2) and Abridged Universal Amplification Primer (AUAP, provided in the kit) for each of the members under the following condition: 94°C for 3 min, followed by 35 cycles of amplification (94°C for 1 min, 57°C for 1 min and 72°C for 1 min), and the final elongation for 10 min at 72°C. The 5′ cDNA fragments were amplified using specific reverse primer 1 (CHS1R1 and CHS2R1) for each CHS; then, the first round of PCR amplification was done with specific reverse primer 2 (CHS1R2 and CHS2R2) and Abridged Anchor Primer (AAP, provided in the kit) under the following conditions: 2 min at 94°C, 35 one-minute cycles at 94°C, 1 min at 55°C, 2 min at 72°C, and finally 7 min at 72°C. Subsequently, the PCR products were used as templates for the nested PCR amplification with the specific reverse primer 3 and AUAP. The PCR amplification was done using the following conditions: 3 min at 94°C followed by 35 one-minute cycles at 94°C, 1 min at 55°C, 1 min at 72°C, and final 10 min at 72°C. Cloning and sequencing of the cDNA were performed using the methods described earlier.

Relative quantitative real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen) from milk thistle in two developmental stages, including early flowering stage (bright purple petals) and mid-flowering stage

(dull purple petals) (figure 1) from the following tissues; petal, flower head, five upper leaves (leave 1) and the related stem (stem 1), the next five upper leaves (leave 2) and the related stem (stem 2). First strand cDNA was amplified by 1 μg of total RNA from each sample as the template using iScript cDNA synthesis kit (Biorad) according to the manufacturer's protocol. Relative quantitative real-time PCR reactions were performed in a 96 well-plate with an iCycleriQ real-time PCR (Biorad) using iQ SYBR Green Supermix (Biorad) to monitor cDNA amplification according to its instructions. The reaction mix (25 μL) contained 1 μL cDNA, 1 μL of each 10 μM forward and reverse primers, 12.5 μL SYBR Green supermix, and sterile distilled water to reach the final volume. Thermal cycling conditions were 95°C for 2 min followed by 45 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and then 72°C for 5 min. Relative quantification was determined by the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001) using 18S rRNA as an internal control. Calibrator for early flowering and mid-flowering stages was stem 2 of the relevant stage. In all the experiments, three independent biological replicates with three experimental repetitions were done for each RNA sample. Analysis of variance for comparing treatment means was determined by least significant difference tests ($P = 0.05$), available in the SAS package (Afifi *et al.* 2003).

Comparative in silico analysis and phylogenetic tree reconstruction

Multiple sequence alignments of the nucleotide and amino acid sequences of *SmCHS3* and *CHSs* from other plant species were done using clustal W. DNASTAR software (Lasergene, USA) was used to predict the deduced amino acid sequences of those CHS and SmCHS3's open reading frames (ORF). The phylogenetic tree was constructed by the unweighed pair group method with arithmetic mean (UPGMA) method (Sokal and Michener 1958) with 1000 replicates. The reliability of each node was established based on bootstrap calculations using MEGA4 software (Tamura *et al.* 2007). Number of covered nucleotide, amino acid and similarity percentage between milk thistle's CHS and Asteraceae family were calculated by GeneDoc software (Free Software Foundation, USA).

Result

Cloning of partial genomic DNA and full-length cDNA of CHS from milk thistle

Fragments of *CHS* genes were amplified from milk thistle using primer pairs F1R1, F1R2, F2R1 and F2R2, yielding two PCR products with the expected size of 746 bp and 592 bp (figure 3 in electronic supplementary material). However, the amplified fragments using F1R1 and F1R2 primer pairs (1200 and 1069 bp) were larger than the expected size (1016 bp and 861 bp, respectively) (figure 1 in electronic supplementary material).

Alignment of the nucleotide and deduced amino acid sequences of F1R2 and F2R1 fragments revealed only about 73% and 70% identity, respectively, indicating the identification of two different members of *CHS* gene family from milk thistle called *SmCHS1* (GeneBank accession no. JN182805) and *SmCHS2* (GeneBank accession no. JN182807), respectively. BLASTn search revealed that *SmCHS1* and *SmCHS2* partial genomic DNA fragments had 85% and 78% similarity to *Callistephus chinensis* and *Chrysanthemum indicum CHS* genes, respectively. Thus, these fragments were used to design gene-specific primers to clone the 5′ and 3′ ends of the full-length cDNA for both *CHS* by 3′RACE and 5′RACE methods. All the amplified fragments were cloned and sequenced. One (950 bp) and two (863 and 600 bp) fragments were amplified by 3′RACE using specific primer pairs of *SmCHS1* and *SmCHS2*, respectively, but only one PCR product of 5′RACE (779 bp) with specific primer pairs of *SmCHS1* was obtained (figure 4 in electronic supplementary material) and used as the template for the nested PCR; which lead to the amplification of the expected fragment (621 bp). Blastn search confirmed that the amplified fragments by 5′RACE and 3′RACE using specific primer pairs of *SmCHS1* were indeed the 5′ and 3′ ends of the *CHS* gene, but the two amplified fragments by 3′RACE using specific primer pairs of *SmCHS2* had no significant similarity to *CHS*. The ATG start codon was followed by GCATCC; thus, the second and third residues were Ala and Ser, which are predominant residues in the corresponding positions in highly-expressed plant genes. A 34-bp untranslated region was the upstream of the start codon (5′UTR) and the coding region was followed by a 133 bp 3′UTR with putative polyadenylation signal (AATAAT) in the position 22 bp downstream from the stop codon. Alignment of full-length cDNA and *SmCHS1* showed only 98% homology in nucleotide and amino acid sequences (figure 2 and table 2 in electronic supplementary material), indicating the identification of another milk thistle's *CHS* called *SmCHS3* (GeneBank accession no. JN182806). The protein encoded by *SmCHS3* had the calculated molecular mass of 45.05 kDa and the isoelectric point of 6.35.

Multiple sequence alignments and phylogenetic analysis of milk thistle's CHS family

Multiple nucleotide and protein sequence alignment of *CHS* gene family from milk thistle and other Asteraceae species revealed 70–98%, 62–73% and 64–83% similarity in the covered nucleotide sequences and 70–94%, 66–73% and 73–93% similarity in the covered amino acid sequences of Asteraceae species CHS family members and *SmCHS1*, *SmCHS2* and *SmCHS3*, respectively (table 2 in electronic supplementary material).

A phylogenetic tree was constructed based on the multiple alignments of full-length cDNA sequences from various plants and *SmCHS3* using the UPGMA method (figure 2A).

The result showed that they were grouped into two distinct clades: one containing the monocotyledonous plants such as *Oryza sativa* and *Zea mays* (family: Poaceae) and *Lilium hybrid* (family: Liliaceae), and another was specific for the dicotyledonous plants as previously reported (Niesbach-Klösgen *et al.* 1987). Dicotyledonous plants were diverged into two parts: one containing Asteraceae family (*Gerbera hybrid*, *Chrysanthemum* × *morifolium*, *Silybummarianum*, *Callistephus chinensis*) and another including other dicot plants (*Malustoringoides* and *Fragaria* × *ananassa* (family: Rosaceae), *Juglansnigra* × *Juglansregia* (family: juglandaceae), *Gossypium hirsutum* (family: Malvaceae), *Lonicera japonica* (family: Carprifoliaceae), *Camellia sinensis* (family: theaceae), *Vaccinium corymbosum* (family: Ericaceae), *Senna alata* and *Senna tora* (family: Fabaceae)). The result revealed a close relationship between the *CHS* from milk thistle and other Asteraceae species, suggesting that they may share common ancestors.

Gene expression pattern of CHS in milk thistle

Transcripts of the *SmCHS*s were detected in all the examined tissues (including petal, flower head, different leaves and diverse parts of the stem) and developmental stages (early and mid-flowering). During early flowering stage, the uppermost expression level of *SmCHS1/SmCHS3* was ob-

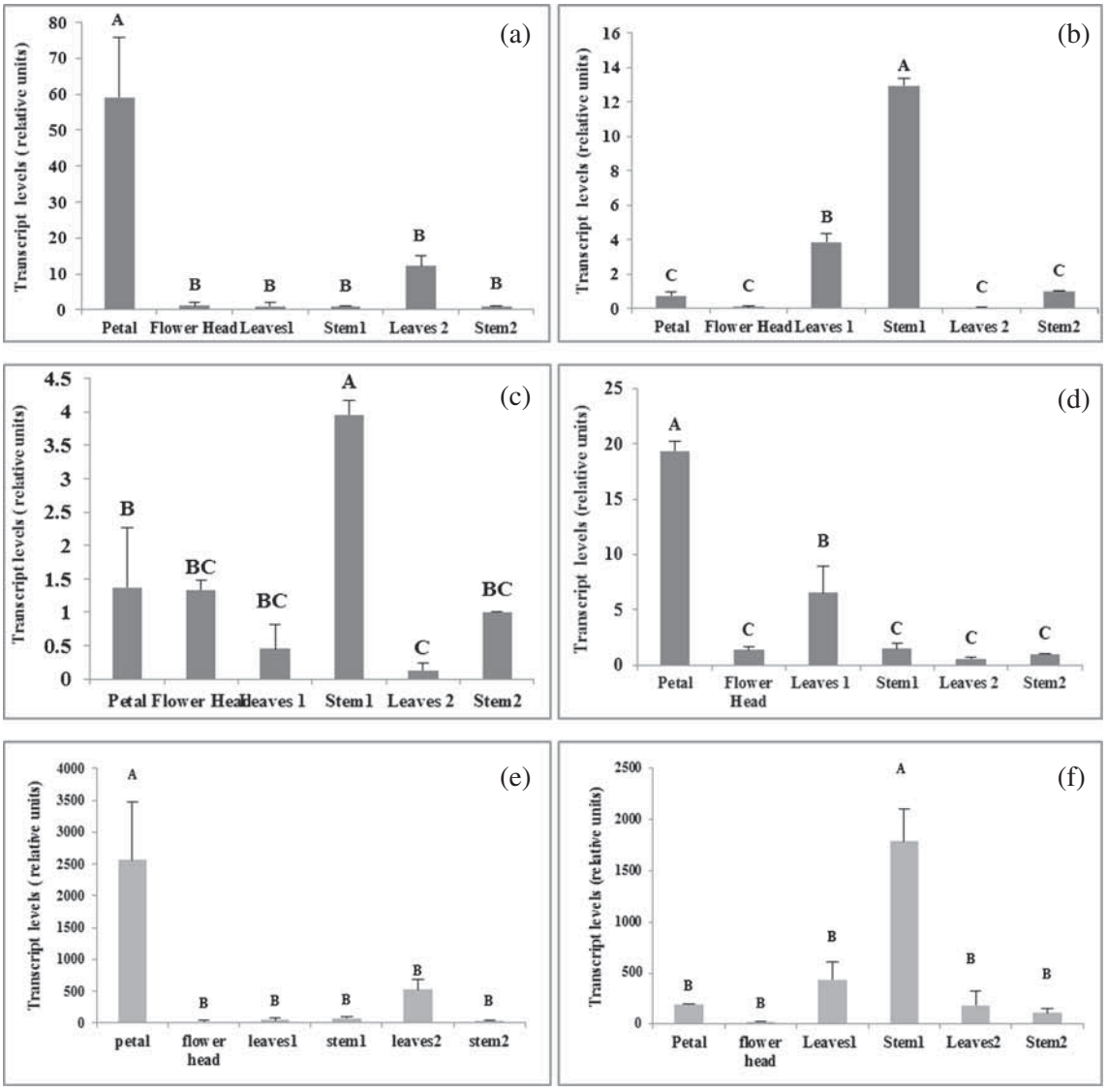


Figure 3. Expression pattern of *SmCHS*s obtained by relative quantitative real-time PCR: (a) *SmCHS1/SmCHS3* expression in early flowering stage (relative to the transcript level of *SmCHS1/SmCHS3* in stem 2). (b) *SmCHS1/SmCHS3* expression in mid-flowering stage (relative to the transcript level of *SmCHS1/SmCHS3* in stem 2). (c) *SmCHS2* expression in early flowering stage (relative to the transcript level of *SmCHS2* in stem 2). (d) *SmCHS2* expression in mid-flowering stage (relative to the transcript level of *SmCHS2* in stem 2). (e) *SmCHS1/SmCHS3* expression in mid-flowering stage (relative to the transcript level of *SmCHS2* in stem 2 of the same stage). (f) *SmCHS1/SmCHS3* expression in mid-flowering stage (relative to the transcript level of *SmCHS2* in stem 2 of the same stage). Columns followed by the same letter are not significantly different at 5% level by LSD test.

served in petals, whereas during mid-flowering, the highest transcript level of *SmCHS1/SmCHS3* was found in stem 1 followed by leaves 1 (figure 3, a&b).

Maximal level of *SmCHS2* transcripts was observed in stem 1 followed by petals in the early flowering stage, whereas the highest level of *SmCHS2* transcript was accumulated in petals followed by leaves 1 in the mid-flowering stage (figure 3, c&d).

Compared to the expression of *SmCHS1/SmCHS3*, the *SmCHS2* was minimally expressed in all the tissues tested. Therefore, the transcription level of *SmCHS2* in stem 2 in each stage was used as the calibrator for *SmCHS1/SmCHS3*. Data revealed that *SmCHS1/SmCHS3* transcript levels were significantly higher than those of *SmCHS2* in petals and stem 1 by about 2550 and 1790 fold in the early flowering and mid-flowering stages, respectively (figure 3, e&f).

Surprisingly, not only the temporal highest levels of expression of *SmCHS1/SmCHS3* and *SmCHS2* in the early flowering stage dramatically declined by the mid-early flowering stage, but also the highest levels of expression of *SmCHS1/SmCHS3* and *SmCHS2* had opposing expression patterns to each other in these stages.

Discussion

Sequencing result of the amplified fragments using F1 R2 primer pairs showed that this *CHS* gene contained a 208-bp intron disrupting cysteine codon (amino acid 64) as previously reported for *CHS* genes from other species (Durbin *et al.* 2000; Harashima *et al.* 2004). Full-length cDNA was identified by overlapping the 3'RACE and 5'RACE sequences whose ORF contained 1239 bp including exon 1 (190 bp) and exon 2 (1049 bp) encoding 63 and 349 amino acid residues, respectively, which was in good agreement with the size of the known *CHS* genes in other plant species (Fliegmann *et al*. 1992; Lei *et al*. 2010; Zhou *et al*. 2011). The strictly conserved CHS active site residues, Cys^{168} His^{307} and Asn^{340} (Ferrer *et al.* 1999) as well as the highly conserved CHS signature sequence, G^{376}FGPG (Suh *et al.* 2000) were found in SmCHS3 (figure 2B). The Phe^{219} and Phe^{269} residues, which are important in determining the substrate specificity of CHS (Jez *et al.* 2002) were all present.

CHS is often highly expressed in flowers, wherein it is associated with the accumulation of anthocyanin pigments (Helariutta *et al*. 1995). High expression of *SmCHS1/SmCHS3* in petals but low expression of *SmCHS2* at the petal production stage (early flowering stage), suggests that *SmCHS1/SmCHS3* are responsible for anthocyanin biosynthesis, as reported in anthocyanin-pigmented tissues such as for *PsCHS1* in *Paeonia suffruticosa* (Zhou *et al.* 2011), *AmCHS1* in *Antirrhinum majus* (Hatayama *et al.* 2006), *CHSA* and *CHSJ* in *Petunia hybrida* (Koes *et al.* 1986), *GCHS1and GCHS3* in *Gerbera hybrida* (Helariutta *et al.* 1995), *CHSD* and *CHSE* in *Ipomoea purpurea* (Durbin *et al.* 2000), *LhCHSA*, *LhCHSB* and *LhCHSC* in Asiatic hybrid lily (Nakatsuka *et al*. 2003).

It is noteworthy that a previous study reported that *CHS* gene expression in organs lacking anthocyanins might be due to the expression of *CHS* genes that are used for the biosynthesis of other secondary metabolites, such as flavones and flavonols in *Gerbera hybrida* (Helariutta *et al.* 1995), Asiatic hybrid lily (Nakatsuka *et al.* 2003), *Dendrobium* orchid (Mudalige-Jayawickrama *et al.* 2005), and *Paeonia suffruticosa* (Zhou *et al.* 2011).

Carrier *et al*. (2003) found no detectable levels of silymarin in flower head in the early flowering stage (bright purple) of development and that it slightly started accumulation in seeds in the mid-flowering stage (dull purple). The highest content of silymarin was obtained from the late flowering (no petal, slight brown seeds) and dehiscing developmental stages (no petal, black seeds). It could be recommended that, not only *SmCHS1/SmCHS3* was strongly expressed in petals to be responsible for anthocyanin accumulation in the early flowering stage, but also it was involved in the biosynthesis of secondary metabolites in the mid-flowering stage (the biosynthetic pathway for some silymarin constituents e.g. silybin and taxifolin is presented in figure 5 in electronic supplementary material). It could also be assumed that the leaves 1 in mid-flowering stage were involved in silymarin biosynthesis and stem 1 transferred the components to flower head.

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