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Molecular cloning and expression profiling of a chalcone synthase gene from *Ginkgo biloba*, *Scutellaria viscidula Bunge* and *Tree peony* (*Paeonia suffruticosa)*

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Key words: Chalcone Synthase (CHS), Molecular cloning, Methyl jasmonate (MeJA), *Ginkago biloba*, *Scutellaria viscidula*, *Tree peony* (*Paeonia suffruticosa*).

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Abstract

Chalcone synthase (CHS, EC 2.3.1.74) is a key enzyme of the flavonoid/isoflavonoid biosynthesis pathway. In this review, the characterization and expression profile of CHS was investigated from *Ginkgo biloba*, *Scutellaria viscidula*, and *Tree peony* (*Paeonia suffruticosa*) because of the CHS role in protecting the plants from harmful effects by synthesizing flavonoid compounds. CHS was isolated and characterized from the same species which was described previously. The cDNA sequence of the CHS gene in *Ginkgo biloba* was 1608 bp with poly (A) tailing, from *Scutellaria viscidula*1649 bp, and from *Tree peony* (*Paeonia suffruticosa*) 1475 bp. cDNA sequence of the CHS gene in *G. biloba* contained a 1173 bp open reading frame (ORF) encoding a 391 amino acid protein, from *Scutellaria viscidula* 1170 bp encoding a 390 amino acid protein, and from *Tree peony* (*Paeonia suffruticosa*) 1185bp encoding a 394 amino acid protein. Sequence alignment and phylogenetic analysis showed that GbCHS, SvCHS and Ps-CHS1 shared high homology from other plants. Active site of the CoA binding, coumaroyl pocket and cyclization pocket in CHS protein of *Medicago sativa* were also found in GbCHS, and SvCHS. Likewise, Ps-CHS1 has all the conserved active sites for the CHS function as well as the family signature. The homology-based structural modeling showed that GbCHS most closely resembled of *M.sativa* (MsCHS2). Real-time (PCR) analysis indicated that GbCHS and Ps-CHS1 had varying expression in different tissues, while expression of SvCHS was induced by MeJA and was the greatest at 12 h. To our knowledge, this is the first report which explains the molecular cloning and expression profiling of a chalcone synthase gene from those species mentioned above.

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Introduction

Considered as one of the oldest living species, *Ginkgo biloba* has grown and furnished in the forests (Mahadevan and Park, 2008). *Ginkgo biloba* is called "living fossil" according to its survival over 170 million years (Sati and Joshi, 2011). The Ginkgo leaf extract has many active components such as ginkgo flavonoids which are essential for plant growth (Yang *et al.*, 2002; Makoi *et al.*, 2010). In plants, flavonoids have several significant functions such as UV protection and defense against pathogens (Jiang *et al.*, 2006; Huang *et al.*, 2010). Chalcone synthase (CHS) is employed as an enzyme for secondary metabolites biosynthesis compounds like flavonoid (Yang *et al.*, 2008). CHS orchestrates the condensation of three acetate remnants derived from malonyl-CoA with p-coumaroyl-CoA to form naringenin-chalcone, which considers the initial committed step that leads to the production of flavonoids, isoflavonoids, anthocyanin and others in plants (Martens and Mithöfer, 2006).

Metabolism pathway of flavonoids will be elucidated gradually as the following; in the pathway new compounds are found out and each of them is found to have specific positive functions in diverse tissues such as UV defense, resistances against pathogens and insects (Winkel-Shirley, 2002). The products of pathway make plants more efficient to resist stressful environments (Yang *et al.*, 2002).

Several recent studies have shown that numerous (CHS) genes were characterized of diverse species such as *Zea mays* (Franken *et al.*, 1991), *Arabidopsis* (Saslowsky *et al., 2000), Bromheadia finlaysoniana* (Liew *et al.*, 1998), *Petunia hybrid* (Napoli *et al.*, 1999) and *Pines* (Fliegmann *et al.*, 1992). All the cloned (CHS) genes were found to belong to multi-gene family (Pang *et al.*, 2005). *G. biloba* is the only species that belongs to the order Ginkgoales (Kvacek *et al.*, 2005). Hence, the detailed study of (CHS) gene in *G. biloba* will be helpful in understanding its evolutional position in the plant kingdom.

Plants, which belong to genus *Scutellaria*, have many medically significant flavonoids (Zheljazkov *et al.*, 2007). Several studies showed that *Scutellaria* flavonoids have medical prosperities against viruses such as (HIV-1) (Kovács *et al.*, 2004) and many types of cancer like prostatic, urothelial and colon cancer in vitro (Ye *et al.*, 2002). Defined as one of the endogenous phytohormone (Moreira *et al.*, 2009), Methyl jasmonate (MeJA) has a critical role in mediating various developmental processes such as root growth, resistance to insects and pathogens (Cao *et al.*, 2012). For example, pal (phenylalanine ammonia lyase), CHS (chalcone synthase), STS (stilbene synthase) and UBGAT (UDP-glucose: flavonoid 7-O-glucosyltransferase) expression is catalyzed by treatment with Methyl jasmonate (MeJA) in *Vitis vinifera L* (Belhadj *et al.*, 2008).

Tree peony (*Paeonia suffruticosa*) is the most popular decorative plant in china because of its big wonderful flower (Mornya *et al.*, 2011). 3-Oglucosidesand 3, 5-di-O-glucosides of pelargonidin, cyanidin and peonidin represent the main anthocyanins in the *Tree peony* (*Paeonia suffruticosa*) family. Further, apigenin, luteolin, kaempferol, quercetin, chrysoeriol and isorhamnetin represent major of flavones as it is shown in the previous studies (Wang *et al.*, 2001; Wang *et al.*, 2005). In this review, a description of the molecular cloning of a CHS from *Ginkgo biloba*, *Scutellaria viscidula*, and *Tree peony* (*Paeonia suffruticosa*) will be investigated. In addition, the expression profile of CHS from the same species, which was mentioned previously, will be examined.

Material and methods

Cloning of the full-length cDNA

Ginkgo biloba

CTB method was used to extract total RNA (Jaakola *et al.*, 2001). RT-PCR and RACE-PCR were carried out using RT-PCR Kit (TaKaRa) and SMART™ RACE cDNA Amplification Kit (Clontech), respectively(Xu *et al.*, 2012; Kashkush *et al.*, 2003). The primers, FGbCHS (5'CCKTCHYTGGAYGCNMGRCARCAC-3') and RGbCHS (5'GGBCCRAANCCRAANARMAC- 3'),

were designed for RT-PCR reaction based on the highly conserved amino acid sequences of CHSs shared by other plants species (Pang *et al.*, 2004). Race-PCR was performed to identify full length cDNA sequence while RT-PCR was used to verify the full-length cDNA sequence (Frohman *et al.*, 1988; Bustin, 2000).

Scutellaria viscidula

Based on the manufacturer's instructions (Tiangen, Beijing,China) of TRIzol reagent, total RNA was isolated (Bilgin *et al.*, 2009) from shoots and blank hairy roots of *S.viscidula*. Using TaKaRa RNA PCR kit ver. 3.0 (TaKaRa, Dalian, China) led to synthesize single-standard of cDNA which was utilized as a template for PCR (Goto-Yamamoto *et al.*, 2002; Hillmann *et al.*, 2009). 3'- and 5'-ends cDNA were obtained by using ASMART RACE cDNA amplification kit (Kashkush *et al.*, 2003). Two primers, DFCHS (5'-TTCATGATGTACCAGCAGGGCTGCT-3') and DRCHS (5'GGAGG (A/C) CTTCCTCATCTCATCCA-3'), were prepared for amplification of *S.viscidula* CHS gene according to the conserved region of CHS genes from different plant species (*S. baicalensis* chs-C, *S.baicalensis* chs-P, S. *baicalensis* chs and *Perilla frutescens* chs) (Lei *et al.*, 2010). RT-PCR was used to amplify cDNA whereas Race- PCR was used to obtain the full-length cDNA of CHS gene (Frohman *et al.*, 1988; Rajeevan *et al.*, 2001).

Tree peony (Paeonia suffruticosa)

The modified cetyltrimethylammonium bromide (CTAB) method was manipulated to extract total RNA (Chang *et al.*, 1993) from total disclosed flower petals. Using M-MuLV reverse transcriptase (Promega) led to synthesize first stand cDNA (Ståhlberg *et al.*, 2004). Degenerated primers for PCR reaction, 5'CA (A/G) CCCAAGTCCAA (A/G) AT(C/T) ACCC3' (forward) and 5' (A/T) CCCCACTC (A/C/G) AG(C/T/G) CCTTC (A/T) CC-3' (reverse), were designed based on the conserved sequence from other cloned CHS genes (Zhou *et al.*, 2011).

Bioinformatic analysis

Ginkgo biloba

The sequences of GbCHS gene were analyzed and calculated with bioinformatics computer tools at following websites (www.ncbi.nlm.nih.gov, www.expasy.org) (Luscombe *et al.*, 2001). The sequence Multi-alignments was carried out by using software Vector NTI™ Suite8 (Huang *et al.*, 2008) for GbCHS. GbCHS and other known CHS from other plant species retrieved from GenBank (Table 1) were aligned with CLUSTALW(1.82) (Kumar *et al.*, 2001). Phylogenic tree was built by the neighbor joining method (NJ) using the software of MEGA 2.0 (Kumar *et al.*, 2001; Saitou and Nei, 1987). The reliability of the tree was measured by bootstrap analysis with 100 replicates (Felsenstein, 1985).

Scutellaria viscidula

The sequences of SvCHS gene were analyzed and calculated with bioinformatics computer tools at the NCBI and Expasy websites (Luscombe *et al.*, 2001). ORF was analyzed by using ORF finder (Kalpesh, 2012). The sequence Multiple-alignments of SvCHS was done by using software Vector NTI™ Suite8 (Lei *et al.*, 2009). SvCHS and other species plant CHSs were aligned with CLUSTALW (1.82) (Li, 2003). Phylogenic tree was built by the neighbor joining method (NJ) with 1000 replicates. The reliability of the tree was calculated by bootstrap analysis using the software of MEGA 3 (Kumar *et al.*, 2004).

Tree peony (Paeonia suffruticosa)

DNAStar was used to determine the sequence assembling (Burland, 1999). The sequences of Ps-CHS1 gene were analyzed and calculated with bioinformatics computer tools at the NCBI and Expasy websites (Luscombe *et al.*, 2001). Multiple sequence alignments of the sequence Ps-CHS1 was performed by using (DNAMAN ver. 6.0.3.99 Lynnon Biosoft). Phylogenic tree were performed by using (DNAMAN ver. 6.0.3.99 Lynnon Biosoft) (Zhou *et al.*, 2011).

Expression profile analysis

Ginkgo biloba

RT-PCR was carried out by using kit (TaKaRa, Japan) with two primers GBCHSF1 (5'-ATGGAAGACTTGGAGGCATTCAGG-3') and GBCHSR1 (5'-TTACTTGTTGCAGGGAACGCTCCT-3'). RT-PCR reaction for (18S) gene was also performed as a control using specific primers 18Sf (5'ATGATAACTCGACGGATCGC-3') and 18Sr (5'CTTGGATGTGGTAGCCGTTT-3'). The PCR products were separated on 1% agarose gels stained with ethidium bromide (10 mg/ml) and gene analysis software package (Gene Company, USA) was used to analyze the quantities of product (Pang *et al*., 2004).

Scutellaria viscidula

MeJA (200 mol/L) was used to treat hairy root cultures, subsequently hairy root cultures were harvested after 6, 12, 24, 48 and 72 h. Total RNA was obtained from treated and non-treated hairy roots. RT-PCR was manipulated using Kit (TaKaRa) with two primers fexSvCHS (5'ATGGTGACAGTTGAAGAATTCCA-3') and rexSvCHS (5'ATTGAGAGGCACACTATGCAGAA-3') for examining the changes in SvCHS expression. Specific primers 18Sf (5'ATGATAACTCGACGGATCG-3') and 18Sr (5'CTTGGATGTGGTAGCCGTTT-3') were used to amplify 18S rRNA gene by applying RT-PCR method. 18S rRNA was used as a control to ensure that equal amounts of total RNA were used in reaction. The PCR products were separated in Goldview-stained 1% agarose gels. Successively, Gel-Pro Analyzer 4.0 software (Media Cybernetics, Bethesda, MD, USA) was used to analyze the measure of gray density of the target bands (Lei *et al*., 2010).

Tree peony (Paeonia suffruticosa)

Total RNA was isolated from petals and different tissues such as leaves, sepals, stamens and carpels. RT-PCR was carried out using kit (TaKaRa) with two primers Ps-CHS1 (forward) (5'AGCAGAGAACAACAAAGGGTCACG-3)' and Ps-CHS1 (reverse) (5'TCAGCACCGACAATAACCGCAG-3') to detect the expression of Ps-CHS1. RT-PCR reaction for amplification beta-Tubulin gene, considered as an internal control, was performed using (5'TGAGCACCAAAGAAGTGGACGAAC-3)' (Forward) and (5'CACACGCCTGAACATCTCCTGAA-3') (Reverse) primers.

Results and Discussion

Cloning of the full-length cDNA

Ginkgo biloba

Full-length cDNA contained 1608bp and 1173 pb (ORF), which encoded a 391 amino acid protein. Tow un-translated region were recognized. 5'untranslated region (upstream) included 79bp, and 3'untranslated region (downstream) had 353bp including the poly (A). Two polyadenylation signals (AATAA) were located in the different downstream position (16bp and 220bp from the stop codon). Polyadenylation signals were very significant to offer a high degree of stability (Pang *et al*., 2004) (See Fig 1).

Scutellaria viscidula

According to sequence analysis, Full-length cDNA of SvCHS contained 1649bp and 1170 pb (ORF) which encoded a390 amino acid protein. Furthermore, there were two un-translated regions 5' (UTR) about (89) bp and 3' (UTR) about (390) bp surrounding the gene with a putative poly(A) signal AATAA in downstream at position 100bp from the stop codon (See Fig 2) (Lei *et al*., 2010).

Tree peony (Paeonia suffruticosa)

The sequencing of DNA revealed that Ps-CHS1 was 1475bp including 5' (UTR) and 3' (UTR) which were 82bp and 208bp respectively with polyadenylation and 1185bp of (ORF) which encoded a 394 amino acid (See Fig 3) (Zhou *et al*., 2011).

Characterization of the deduced protein

Ginkgo biloba

Isoelectric point was calculated by using the pI/Mw Tool at www.expasy.org (Wilkins *et al*., 1999), and the molecular weight of the deduced GbCHS polypeptide was predicted to be about 6.28 and 42 kDa, respectively (Pang *et al*., 2004). According to Xu *et al*. (2008) the deduced GbPal had high similarity with other CHSs from different species of plants. This

result was obtained by using BlastP2.2.3 and the multi-alignment with Vector NTI, hence, BlastP2.2.3 (National Center for Biotechnology Information databases) and the multi alignment with vector NTI were used to reveal that the deduced GbCHS had considerable high homology with CHSs from various species of plants. Moreover, there was high homology between the amino acid sequences of GbCHS with CHSs from other corresponding plants. The percentage of amino acid sequence of GbCHS was 87% which was identical to CHSs from *Pines* (*Pinus pinaster*, *P. strobes* and *P. sylvestris*), 86% identical to CHS from *Picea mariana*, 84% identical from *Glycine max*, 82% identical from *Juglans nigra*, 81% identical from *P. hybrida*, and 80% identical from *M. sativa*. Based on these results, there was a relative relationship with CHSs from other gymnosperm plant (Pang *et al*., 2004). Several activities sites for CHS in different plant species were observed in GbCHS. For example, T132, M137, F215, I254, G256, F256, and P375, which are residues of cyclization pocket, were found in GbCHS. C164, H303 and N336 were defined as catalytic sites whereas residues of cumaroyl pocket were represented by S133, E192, T194, T197 and S338 (See Fig 4). K55, R58 and K62, named as a CoA binding active sites, were detected in the GbCHS (Pang *et al*., 2004). These active sites and positions mentioned above were the same with those of MsCHS2 (Jez and Noe, 2000).This indicate to GbCHS belonged to CHS family.

Table 1. CHS proteins used in phylogenetic analysis (Pang *et al*., 2004).

A CHS GENE FROM *GINKGO BILOBA*

TABLE 1 CHS proteins used in the phylogenetic analysis

Sequence name	Protein accession numbers in GenBank	Species
AmCHS	CAA27388	*Antirrhinum majus* (snapdragon)
AtCHS	NP_196897.1	*Arabidopsis thaliana* (thale cress)
BfCHS	AAB62875.1	*Bromheadia finlaysoniana*
CsCHS2	BAA05641	*Camellia sinensis* (black tea)
CgCHS1	CAA10641	*Casuarina glauca* (swamp oak)
CsCHS2	BAA81664	*Citrus sinensis*
Cmachs1	CAA38456	*Glycine max* (soybean)
JnCHS2	CAA64366.1	*Juglans nigra* × *Juglans regia*
LeCHS1	CAA38980.1	*Lycopersicon esculentum* (tomato)
MdCHS	BAB92996	*Malus* × *domestica* (apple tree)
MsCHS2	AAB41559	*Medicago sativa* subsp.*sativa*
PhCHS	CAA32737	*Petunia* × *hybrida*
PmCHS2	AAF35890	*Picea mariana*
PpCHS	AAN87170	*Pinus pinaster*
PstCHS	CAA86077	*Pinus strobus*
PsyCHS	CAA43166	*Pinus sylvestris* (Scots pine)
PsCHS4	BAA22042	*Pisum sativum* (pea)
PtmCHS	BAA01075	*Pueraria montana* var. *lobata* (kudzu vine)
RhCHS	BAC66467	*Rosa hybrid* cultivar "Kardinal"
RiCHS	AAK15174	*Ribes sativa*
SbCHS8	AAL49965.1	*Sorghum bicolor* (sorghum)
TsCHS1	AAA18176.1	*Trifolium subterraneum*
VvCHS3	BAB84111	*Vitis vinifera*
ZmC2	CAA42764.1	*Zea mays*
ZmWhp	CAA42763.1	*Zea mays*
AtKAS	NP_199441	*Arabidopsis thaliana*

Table 2. Percent identity and similarity of Ps-CHS1 amino acid sequence with CHS genes from different plant species (Zhou *et al*., 2011).

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

Scutellaria viscidula

Isoelectric point was calculated and molecular mass of the deduced SvCHS was predicted to be about 42.56 KDa and 5.79, respectively (Lei *et al*., 2010). BlastP2.2.3 was used to confirm that amino acid sequences of SvCHS had high similarity with CHSs

from other corresponding plants (Cameron and Williams, 2007). The percentage of amino acid sequence of SvCHS was 88% identical to *Antirrhinum majus*, 87% to *Misopates orontium* and *Perilla frutescens*. Regarding *S. baicalensis*, the percentages identity were (CHS: 97% identical, CHS-P: 95% identical, CHS-C: 94%), indicating that SvCHS was considered as member from CHS protein family (Lei *et al*., 2010). Using alignment of full-length sequence with Vector NTI sutie8.0 showed that the active center of the CHS was formed by three highly conserved residues Cys164, His304 and Asn340 (Jez and Noel, 2000) (See Fig 5). In addition, SvCHS had binding site for malonyl-CoA at 313-330 positions. N-myristoy-lation site and myristoy-lated had a role in the stabilization of protein and binding the hydrophobic core. In accordance with the presence of the conserved amino residues SvCHS might stimulate the synthesis of chalcon in the *S.visidula* (Lei *et al*., 2010).

Tree peony (*Paeonia suffruticosa*)

Isoelectric point was calculated, and the molecular mass of the deduced Ps-CHS1 has been predicted to be about 43.3 KDa and 6.19, respectively (Zhou *et al*., 2011). The percentage of sequence identity and similarity for Ps-CHS1 with CHSs from other plant species showed in Table 2 (Ferrer *et al*., 1999). Based on analysis of the deduced amino acid sequences revealed that the protein of Ps-CHS1 shared high degree of identity (86-91%) with CHS sequences obtained from various plant species, such as *Populus alba*, *Citrus sinensis*, *Camellia sinensis*, *Rosa hybrid*, *Malus* × *domestica* and *Glycine max*. Therefore, PsCHS1 belongs to the CHS family (Table 2). Ps-CHS1 had several active sites for the CHS function "RLMMYQRLMMYQQGCFAGGTVLR" (156 to 172) and the family signature "GVLFGFGPGL" (368 to 377) (Ferrer *et al*., 1999; Kim *et al*., 2002). Regarding the active amino acid residues in Ps-CHS1, seven amino acid residues of the cyclization pocket (Thr1 Met137, Phe215, Ile254, Gly256, Phe265 and Pro375), three catalytic triad sites, five residues of coumaroyl (Ser133, Glu192, Thr194, Thr197 and Ser338) pocket. Three CoA was used as a binding for active sites. Described as the most important active-site amino acid residues, Cys164, Phe215, His303 and Asn336 (See Fig 3) were responsible for the reaction of multiple decarboxylation and condensation (Ferrer *et al*., 1999; Schröder *et al*., 1998).

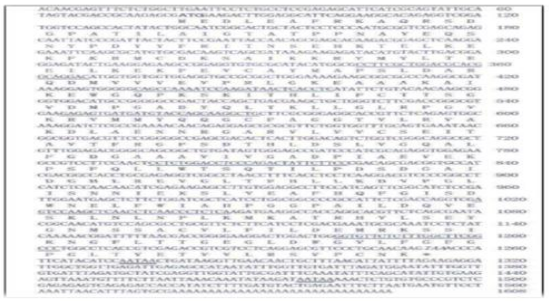

Fig. 1. shows Nucleotide and deduced amino acid sequences of the full-length cDNA of (GbCHS). (ATG) and (TAA), represented start and stop codon respectively, were bolded and bolded italically. The putative polyadenylation signals were recognized by double underline. The primers, which were used for cloning, were underlined (Pang *et al*., 2004).

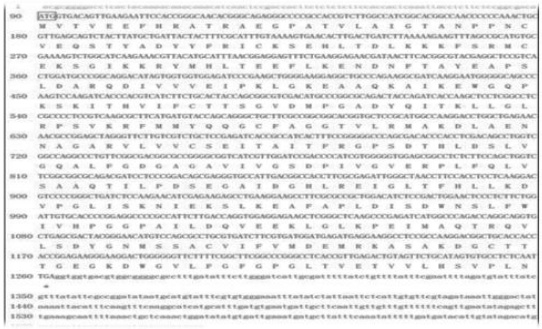

Fig. 2. Full-length cDNA sequence and deduced amino acid sequence of SvCHS. The start codon (ATG) and stop (TGA) codon was marked with boxed and asterisks respectively. The coding sequence of SvCHS is shown in capital letterswhile the 5'- and 3'-untranslated regions are shown in normal letters (Lei *et al*., 2010).

Three-dimensional model of CHS

Ginkgo biloba

The homology-based 3-D structural of GbCHS was performed by using the program SWISSMODEL (Schwede *et al*., 2003). Three dimensional molecular Viewer and WebLab Viewer Lite were applied to perform the three dimensional structure and structural analyses (Wu *et al*., 2012). The structures

of monomer and homomer for GbCHS were designed theoretically against the template of CHS2 of *Medicago sativa* (alfalfa) by using crystallography (Ferrer *et al*., 1999). GbCHS and MsCHS2 had 11 a-helices which were bound by 15 B-sheets (See Fig 6a, b) (Austin and Noel, 2003). As it is shown in the (See Fig 6c) symmetric dimmer is formed by tow monomers (Pang *et al*., 2004). The active sites of K55, R58 and K62, formed CoA binding tunnel, appeared in the homodimer (Ferrer *et al*., 1999; Austin and Noel, 2003) This result denoted that the GbCHS and MsCHS had the same active site, suggesting that GbCHS and MsCHS might have similar function (Pang *et al*., 2004).

Fig. 3. Nucleotide and deduced amino acid sequences of the full-length cDNA of Ps- CHS1. The small letters represented sequence whereas the capital letters were coding sequence. The initiation (ATG) and termination codons (TGA) were underlined. Thr132, Met137, Phe215, Ile254, Gly256, Phe265 and Pro375 were framed. Cys164, His303 and Asn336 were shaded. The (*) indicated to the residues of coumaroyl pocket, including Ser133, Glu192, Thr194, Thr197 and Ser338. The family signatures of chalcone synthase (RLMMYQQGCFAGGTVLR and GVLFGFGPGL) were marked by double underline. The CoA binding active sites such as Lys55, Arg58 and Lys62 were italic and bold (Zhou *et al*., 2011).

Scutellaria viscidula

The (3D) structural modeling of the SvCHS protein was analyzed by using Swiss-Modeling and (3D) structure was visualized by using the WebLab Viewer Lite 4.2 (Wu *et al*., 2012; Arnold *et al*., 2006). The secondary structure of SvCHS was shown in (Fig 7) which consisted of random coils, a-helixes and extended strands (Lei *et al*., 2010).The motifs, supported the biological functions in different proteins, occurred in the coiled-coil structure of SvCHS. Based on the analysis, the SvCHS contained the conventional molecular structure of CHS and demonstrated similarities with data stemming from the experiment of *M. savita* CHS (Jez and Noel, 2000; Ferrer *et al*., 1999).

Fig. 4. Multiple sequence alignment of GbCHS and other plant CHS proteins. Table 1 containsthe abbreviations for aligned CHS proteins and their accession numbers in GenBank. The completely identical amino acids were marked with black foreground and thick gray background, conservative amino with white foreground and light gray background, Block of similar amino acids with white foreground and thick gray background, weakly similar amino acids with black foreground and light gray background and non-similar amino acids with black foreground and white background. The active sites for cyclization pocket were boxed. The catalytic sites were labeled with * (Pang *et al*., 2004).

Tree peony (*Paeonia suffruticosa*)

Swiss-Modeling was used to determine the (3D) structural modeling of the Ps-CHS1 protein (Schwede *et al*., 2003) using the crystal structure of CHS from *Medicago sativa* (alfalfa) as template. The similarity between 3D structure of Ps-CHS1 and template was 82.86% which, in turn, contributes to positive identification of its CHS identity (See Fig 8) (Zhou *et al*., 2011).

Fig. 5. Multiple sequence alignment of SvCHS and other plant CHS proteins. Identical sites are shown in white letters on a black background, conserved sites in white letters on a gray background and other sites in black letters on a white background. The highly conserved catalytic domain motif Cys-His-Asn, an N-myristoylation motif gvlfgf and a malonyl-CoA binding motif veeklglkpeimaqtrq are marked by box (Lei *et al*., 2010).

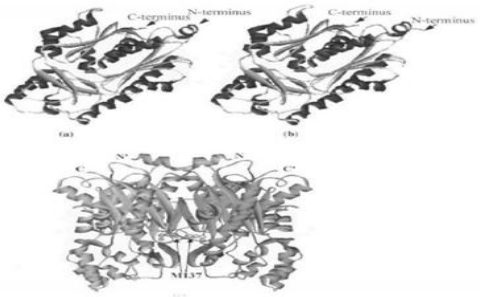

Fig. 6. The three-dimensional models of MsCHS2 (3b for monomer) and the deduced GbCHS (3a for monomer and 3c for homodimer) (Pang *et al*., 2004).

Phylogenetic analysis

Ginkgo biloba

Beta-keto-acyl- ACF synthase from *Arabidopsis thalinna* (Genbank accession NF_199441) (Table 1) was used to examine the relationship between GbCHS and different CHS proteins. The building of phylogenetic tree was done depending on the deduced amino acid sequence of GbCHS and CHS from different plants. Based on analysis of result, there were significant features demonstrated in all the species from these plants. Regarding the first feature, all CHS proteins from family signature sequence were derived from common ancestor. It was not important whether the CHS proteins belonged to any kind of plants, monocot or dicot. In regard to the second feature, the CHS sequence composed several recognizable species-specific clusters. For instance, the cluster formed between *G.biloba* and the other different species from gymnosperm such as *Pines* and *Picea* revealed that *G.biloba* has close relationship with gymnosperm species more evident than its counterpart in angiosperm species (Pang *et al*., 2004) (See Fig 9). It was noticeable that leguminous species such as *G. max*, *Pueraria montana var*, *lobata*, *Trifolium subterraneum*, *M.sativa* and *P. sativum*, were categorized into a cluster. As it was shown from the tree, Rosaceae species including *Malus* × *Domestica*, *Rosa hybrid* and *Rubus idaeus* constituted a cluster. Moreover, Solanaceae species like *tomato* and *Petunia* formed a cluster (O'Neill *et al*., 1990) (See Fig 9). The species which belong to Gramineae such as *Z. mays* and *S. bicolor* formed a cluster in the phylogenic tree. Regarding the third feature, the genetic distance was shorter between the different species such as *Camelia sinensis*, *Fulans nigra* × *Juglans regia* and *Vitis vinifera* based on the phylogenetic tree (See Fig 9). According to the aforementioned result, GbCHS is a homologue with other known CHS gene and CHS protein (Pang *et al*., 2004; Goto-Yamamoto *et al*., 2002; Claudot *et al*., 1999; Takeuchi *et al*., 1994).

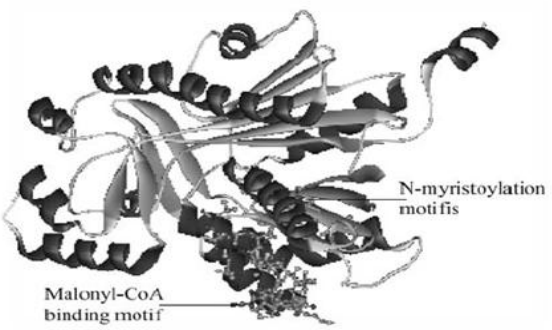

Fig. 7. 3D structural model of SvCHS. Random coils are marked in silver. Selected important motifs are indicated (Lei *et al*., 2010).

Scutellaria viscidula

There were many studies applied on the CHS gene sequences of diverse plants and bacteria. These studies took into consideration the genetic engineering of flavonoids. The CHS genes were aligned in order to investigate the relationships among the CHS genes in different plants (Jiang and

Cao, 2007) (See Fig 10). There was a close relationship between *S. viscidula* and *S.baicalensis* because these species belonged to the same genus (Lei *et al.*, 2010).

Fig. 8. The computing 3D structure of Ps-CHS1 (Zhou *et al.*, 2011).

Tree peony (*Paeonia suffruticosa*)

Phylogenetic analysis showed that monophyletic group belonged to dicotyledons was constituted by CHS proteins, whereas CHS proteins from monocotyledons were grouped as a cluster into another separate clade (See Fig 11) (Zhou *et al.*, 2011). This result matched that of Nakatsuka *et al. (2003)* research. The cluster of dicotyledon CHSs, which contained Ps-CHS1, was segregated into several subgroups according to the various plant species. *Abelmoschus manihot* and *Gossypium hirsutum*, belonging to the Malvaceae family appeared in the same subgroup, while Rosaceae family such as *R. hybrid* and *Malus* × *Domestica* showed in another subgroup (See Fig 11). This result strongly suggests that Ps-CHS1 has a homologue of the CHS gene and protein (Zhou *et al.*, 2011).

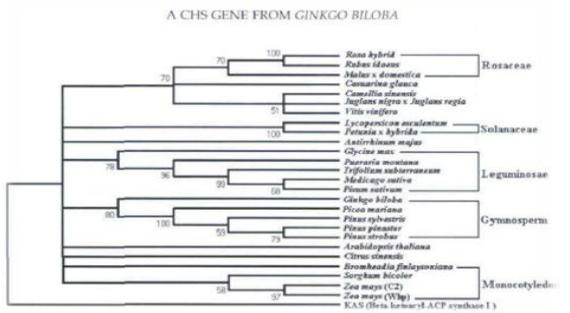

Fig. 9. Neighbor-joining phylogenetic tree of the complete sequences of GbCHS and other 25 CHS proteins was built by using AtKAS as out group (shown in Table 1). Sequences were identified by the names of species. The numbers at each node represented the bootstrap values (Pang *et al.*, 2004).

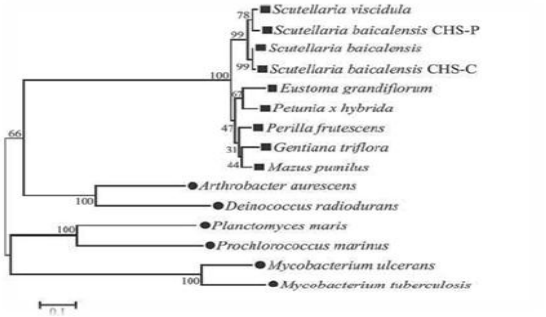

Fig. 10. phylogenetic analyses of plant and bacterial CHS. Neighbor-Joining method was used for constructing of phylogenetic tree (based on 1000 bootstrap replicates) using MEGA3 software; the bootstrap values are shown on the branches. Plant species are marked by ( ) and bacterial species by (*) (Lei *et al.*, 2010).

Expression profile of CHS

Ginkgo biloba

Total isolated RNAs from leaf tissues, stem and root were used for molecular investigation (Liao *et al.*, 2004). RT-PCR was used to detect the expression of CHS (Deepak *et al.*, 2007). Thus this method (RT-PCR) was employed to investigate expression of GbCHS. As it is portrayed in the (Fig 12), there was no expression of GbCHS in the root, whereas, the stem and leaf tissues had an expression of GbCHS (Pang *et al.*, 2005).

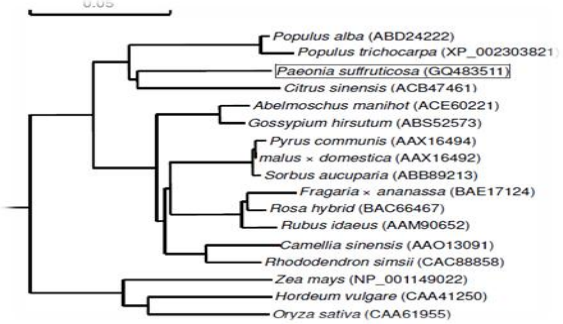

Fig. 11. A phylogenetic tree was constructed based on the deduced amino acid sequences of different CHSs. The Ps-CHS1 protein is boxed (Zhou *et al.*, 2011).

Scutellaria viscidula

Flavonoid baicalin increase by using MeJA at short-term exposure (Wang *et al.*, 2008), while plant cell

growth inhibits when MeJA is used at long- term exposure (Ananieva and Ananiev, 1997). CHS expression was simulated and modulated by using MeJA (Belhadj *et al.*, 2008; Creelman *et al.*, 1992). Therefore, the expression of SvCHS was the greatest after 12h treatment with MeJA followed by progressive decrease in the level of stimulation (See Fig 13). Based on this result, the expression of SvCHS was sensitive for MeJA. Thus, CHS activity and accumulation plant secondary metabolites in the *S. viscidula* would be enhanced in the presence of MeJA (Lei *et al.*, 2010). This result came agreement with what was observed by Sánchez-Sampedro *et al.* (2005). MeJA was employed to enhance CHS activity and boosted the level of silymarin in cell cultures of *Silybum marianum (*Sánchez-Sampedro *et al.*, 2005).

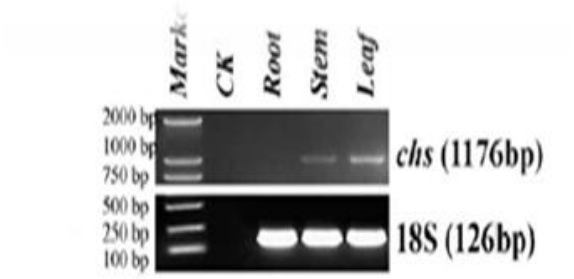

Fig. 12. Expression profiles of GbCHS in different tissues. "Marker, DNA molecular weight marker DL2000; CK, negative control in RT-PCR without template" (Pang *et al.*, 2005).

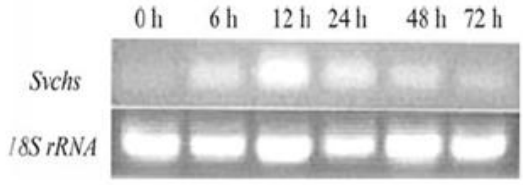

Fig. 13. Expression profiles of SvCHS following treatment by MeJA for different times. Upper part SvCHS expression, lower part 18S rRNA levels used to normalize the amount of template for PCR reactions (Lei *et al.*, 2010).

Tree peony (*Paeonia suffruticosa*)

As shown in (Fig 14), the expression of Ps-CHS1 appeared with high levels in petals pigmented by anthocyanin, moderate levels in sepals, and low and lowest levels were in leaves and carpels (Zhou *et al.*, 2011). Ps-CHS1 expression in petals (See Fig 15) was enhanced gradually to be maximal in stage 6. These results referred that CHS gene, cloned previously, was expressed in a specific tissue (Hua *et al.*, 2004).

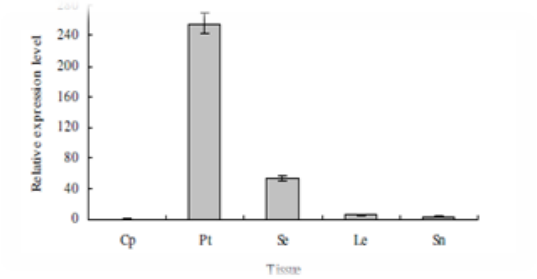

Fig. 14. Expression profiles of Ps-CHS1 in different tissues collected at full opening stage. RT-PCR analyses were carried out using total RNA from carpels (Cp), petals (Pt), sepals (Se), leaves (Le) and stamens (Sn). The internal control was Ps-Tubulin whereas the expression of Ps-CHS1 in Cp was used as a calibration standard (Zhou *et al.*, 2011).

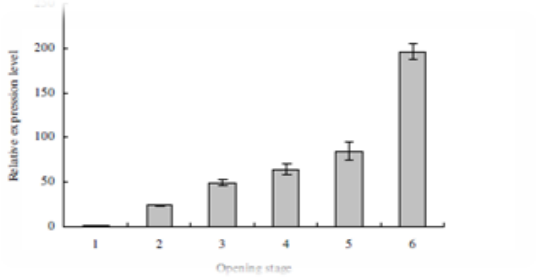

Fig. 15. Expression profiles of Ps-CHS1 in petals at different floral developmental stages. RT- PCR was manipulated using total RNA from petals at each floral developmental stage (1 to 6). Ps-Tubulin was used as an internal control. The calibration standard was Ps-CHS1expressionin petals at stage 1(Zhou *et al.*, 2011).

Conclusions

To summarize, cloning and characterization of GbCHS, SvCHS and Ps-CHS1 have been elucidated, respectively. The protein encoded by these genes GbCHS, SvCHS, and Ps-CHS1 showed high similarity with others known CHS proteins. Based on the structural similarities, GbCHS, SvCHS, and Ps-CHS1 had an important role in the metabolic pathway from different species of plants. RT-PCR showed various

transcript abundances for GbCHS, SvCHS, and Ps-CHS1 genes in specific tissues. Interestingly, these results have presented a distinct idea about regulating genes involved in flavonoid biosynthesis. Finally, flavonoids in *Ginkgo biloba*, *Scutellaria viscidula*, and *Tree peony* (*Paeonia suffruticosa*) have significant functions as a health-protecting natural product.

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