Full Length Research Paper

Cloning and characterization of two novel purple pepper genes (*CHS* and *F3H*)

Ming-hua Deng^{1,2}, Jin-fen Wen³, Jin-long Huo⁴, Hai-shan Zhu², Xiong-ze Dai¹, Zhu-qing Zhang¹, Hui Zhou² and Xue-xiao Zou¹*

¹Institute of Vegetable Crops, Hunan Academy of Agricultural Science, Changsha 410125, China.
²College of Horticulture and Landscape, Yunnan Agricultural University, Kunming 650201, China.
³Faculty of Modern Agricultural Engineering, Kunming University of Science and Technology, Kunming 650224, China.
⁴Faculty of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China.

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The complete coding sequences (CDS) of "Yunnan Purple Pepper No.1" (Capsicum annuum L.) CHS and F3H genes were amplified using the reverse transcriptase polymerase chain reaction based on the conserved sequence information of some Solanaceae plants and known highly homologous pepper ESTs. The nucleotide sequences analysis of these two genes revealed that pepper CHS gene encodes a protein of 402 amino acids that has high homology with the CHS-like protein of six species: Nicotiana tabacum (92%), Rhododendron simsii (91%), Petunia × hybrida (91%), Solanum tuberosum (90%), Vitis vinifera (90%) and Camellia chekiangoleosa (92%). Sequence analysis of the second gene revealed that the pepper F3H encodes a protein of 365 amino acids that has high homology with the proteins of eight species: N. tabacum (89%), Petunia x hybrida (88%), S. tuberosum (87%), Solanum lycopersicum (88%), Litchi chinensis (82%), Actinidia chinensis (81%), Citrus maxima (81%) and V. vinifera (82%). The tissue expression analysis indicated that the pepper CHS gene was over-expressed in pericarp, moderately in stem and flower, weakly in leaf and placenta, and hardly expressed in root and seed. The pepper F3H gene was over-expressed in pericarp; weakly in leaf, flower and seed, and hardly expressed in root, stem and placenta. Our experiment established the primary foundation for further research on these two pepper genes.

Key words: Pepper, CHS, F3H, tissue expression analysis.

INTRODUCTION

The chalcone synthase (CHS) is an important enzyme in anthocyanidins biosynthesis. It catalyzes the first committed step of the condensation of one molecule of 4-coumaroyl-COA with three molecules of malonyl-COA to form naringenin chalcone. Naringenin chalcone is the central intermediate to give flavonoids (Holton and Cornish, 1995; Schröder, 1997; Shirley, 2002), which are important for the pigmentation of flowers and other parts of plants. Several *CHS* cDNA and genomic clones have been isolated from a number of plant species, and the structure and reaction mechanism of higher plant *CHSs* proteins have been extensively studied (Ferrer et al., 1999; Suh et al., 2000; Jez and Noel, 2000; Niesbach-Klosgen et al., 1987; Koes et al., 1989).

Flavanone 3-hydroxylase (F3H) is one of the 'key' enzymes acting at the bifurcation of the anthocyanin and flavonols branches, which catalyzes the stereospecific hydroxylation of (2S) -eriodictyol and (2S)-naringenin to form 3*R*)-dihydroquercetin (2*R*, and (2R, 3*R*)-dihydrokaempferol, respectively (Britsch and Grisebach, 1986; Forkmann et al., 1980; Britsch et al., 1981: Heller Forkmann, and 1993). These dihydroflavonols serve as intermediates for the biosynthesis of anthocyanidins (Holton and Cornish, classified 1995). F3H soluble is as а 2-oxoalutarate-dependent dioxygenase based on its requirements for 2-oxoglutarate, molecular oxygen, ferrous iron (Fe²⁺) and ascorbate. More F3H genes have

^{*}Corresponding author. Email: Pepper_breed@yahoo.cn.

been cloned and characterized from a variety of plant species, such as *Arabidopsis thaliana* (Pelletier and Winkel-Shirley, 1996), *Ginkgo biloba* (Shen et al., 2006) *Hordeum vulgare* (Meldgaard, 1992), *Malus* sp. (Davies, 1993), *Medicago sativa* (Charrier et al., 1995), *Perilla frutescenes* (Gong et al., 1997) and *Zea mays* (Deboo et al., 1995).

Due to the involvement of their products in many biological processes (ultraviolet radiation protection, flower coloration, antimicrobial activity, interspecies interactions, plant defense and medicinal properties), we decided to isolate the pepper *CHS* and *F3H* genes. "Yunnan Purple Pepper No.1" is a pepper variety with high content of anthocyanidins, which was selected from a Yunnan local pepper resources, but the pepper *CHS* and *F3H* genes associated with the biosynthesis anthocyanidins in that Chinese species have not been reported yet.

In the present work, we isolated the purple pepper genes encoding *CHS* and *F3H* owing to their high homology among Solanaceae plant species. We then analyzed the resulting sequences and established their tissue expression distribution.

MATERIALS AND METHODS

Samples collection, RNA extraction and first-strand cDNA synthesis

Yunnan Purple Pepper No.1 was grown in experimental fields of Yunnan Agricultural University. Fresh tissues (root, stem, leaf, flower, pericarp, placenta and seed) for RNA isolations were frozen in liquid nitrogen and stored at -80 °C until further use. Total RNA was extracted using the RNAiso Plus (TaKaRa, Dalian) according to the manufacturer's instructions. To remove genomic DNA contamination, total RNA was digested with RNase-free DNase I (TaKaRa, Dalian). Three micrograms of RNA were reverse transcribed with oligo (dT)₁₈ primer and M-MLV reverse transcriptase (Invitrogen, USA). The efficiency of reverse transcription was checked on 2% agarose gels stained with ethidium bromide.

Isolation of the pepper CHS and F3H genes

The real-time polymerase chain reaction (RT-PCR) was performed to isolate these two pepper genes using the cDNAs from different tissues above. The primers for pepper CHS and F3H gene isolation were designed based on the conserved coding sequences information from some Solanaceae CHS and F3H genes and their highly homologous pepper ESTs sequences. The primers for 5'-CCAGCTAGTTGGTATTTCT-3' CHS were: pepper and 5'-TAGTCACCCAGTTTATTCG-3' and the primers for pepper F3H gene were: 5'-ATAGAAATGCCACCTTCAT-3' and 5'-TTAAGCAAGAATTTCCTCAAT-3'. RT-PCR was carried out as previously described (deng et al., 2011). After the PCR, the gene product was cloned into pMD18-T vector (TaKaRa, Dalian) and sequenced bidirectionally with the commercial fluorometric method. At least five independent clones were sequenced.

Bioinformatics analysis

Sequence analysis of pepper CHS and F3H genes were performed

using softwares in NCBI (http://www.ncbi.nlm.nih.gov) and ExPaSv (http://www.expasy.org). The cDNA sequences were predicted using the GenScan software (http://genes.mit.edu/GENSCAN.html). Putative protein theoretical molecular weight (Mw) and isoelectric point (pl) prediction, signal peptide prediction, subcellular localization prediction and transmembrane topology prediction were performed using the Compute pl/Mw Tool (http://us.expasy.org/ tools/pi_tool.html), SignalP 3.0 server (http://www.cbs.dtu.dk/ services/SignalP/), PSort II (http://psort.hgc.jp/), TMHMM-2.0 server (http://www.cbs.dtu.dk/ services/TMHMM-2.0/), respectively. The Blastp program and Conserved Domain Architecture Retrieval Tool were used to search for similar proteins and conserved domain, respectively (http://www.ncbi.nlm.nih.gov/Blast). The alignment of the nucleotide sequences and deduced amino acid sequences were computed using ClusterX, and the phylogenetic trees were computed using the ClustalX and Mega 4.0 softwares with standard parameters. Secondary structures of deduced amino acid sequences were predicted with SOPMA (http://npsa-pbil.ibcp.fr/). The 3D structures were predicted based on the existing 3D structures by the amino acids homology modeling on swiss server (http://swissmodel.expasy.org/).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as previously reported (Deng et al., 2011). The housekeeping gene *Actin* was selected as a positive control. Control primers used were 5'-TGCAGGAATCCACGAGACTAC-3' and 5'-TACCACCACTGAGCACAATGTT-3'. The primers used were the same as those used for isolation RT-PCR above.

RESULTS

Cloning and identification of pepper CHS and F3H cDNA

Using different tissue cDNAs, the RT-PCR products for the pepper *CHS* and *F3H* genes were 1296 and 1104 bp (Figure 1). These cDNA nucleotide sequences analysis using the BLAST software at NCBI server revealed that pepper *CHS* and *F3H* genes were not homologous to any of the known pepper genes and they were then deposited into the GenBank database (Accession No. JN808444 and JN808445). The sequences prediction were carried out using the GenScan software and results showed that the 1209 and 1098 bp cDNA sequence represent two single genes which encoded 402 and 365 amino acids, respectively. The complete coding sequences (CDS) and the encoded amino acids are presented in Figures 2 and 3.

Physical and chemical characteristics of pepper CHS and F3H

The theoretical pl and Mw of pepper *CHS* and *F3H*, computed using the Compute pl/Mw Tool, were 5.81, 5.31, 44317 and 41108, respectively. The signal peptide prediction performed by SignalP 3.0 on the basis of a combination of several artificial neural networks and



Figure 1. RT-PCR result for pepper *CHS* and *F3H* genes. M, DL2000 DNA marker; 1, PCR product for pepper *CHS* gene; 2, PCR product for pepper *F3H* gene.

ATGGTGACCGTGGAGGAGGTTCGAAGGGAACAACGCGCCAAGGGACCAGCCATCATGGCCATTGGCACGGCGACTCCTTCAAACTGT MUTUEEUR REORAKG PATIMAIG TATPS NC GTTGATCAAGCCACCTATCCTGATTATTACTTTCGAATCACTAATAGCGAACATATGACTGAGCTTAAGGAAAAATTTCAACGCATGTGT U D Q A T Y P D Y Y F R I T N S E H M T E L K E K F Q R M C GATAAATCAATGATTAAGAAGAGGTATATGCATTTAACAGAAGAAATTCTTAAAGAAAATCCTAATATTGTGAATATATGGCTCCTTCT D K S M I K K R Y M H L T E E I L K E N P N I C E Y M A P S CTTGATGCTAGGCAAGATATAGTGGTGGTTGAAATTCCAAAACTTGGCAAAGAAGCAGCCAAAAAGGCTATTAAAGAATGGGGCCCAGCCC L D A R Q D I V V V E I P K L G K E A A K K A I K E W G Q P AAATCGAAGATTACCCATTTGGTGTTTTGCACTACTAGTGGTGTGGACATGCCCGGGGCTGACTACCAGCTCACTAAGCTTCTTGGGCTT K S K I T H L V F C T T S G V D M P G A D Y Q L T K L L G L R P S V K R L M M Y O O G C F A G G T V I R L A K D L A E N AACAAGGGAGCTCGAGTCCTTGTTGTTGCTCTGAAATAACTGCAGTTACTTTCCGTGGCCCAAGTGATACACACTTAGATAGTATGGTT NKGARULUUCSEITAUTFRGPSDTHLDSMU GGACAAGCACTCTTTGGTGATGGGGCGGCGCGCACTCATTGTAGGTTCAGATCCATTACCAGAGGTTGAAAqGCCTTTGTTTGAGCTTGTT G Q A L F G D G A A A L I V G S D P L P E V E R P L F E L V TCTGCGGCCCAAACTCTTCTCCCTGATAGCGAAGGCGCTATAGATGGTCACCTTCGTGAAGTTGGGCTAACATTTCACTTACTCAAAGAT S A A O T L L P D S E G A I D G H L R E V G L T F H L L K D GTTCCTGGATTGATCTCAAAGAATATCGAGAAGAGTTTGATAGAAGCATTCCAACCATTGGGGATTTCTGATTGGAACTCTATCTTCTqG U P G L I S K N I E K S L I E A F 0 P L G I S D W N S I F W ATCGCTCACCCTGGCGGGCCAGCAATCCTCGACCAAGTTGAATTAAAGTTGGGCCTAAAGCTCGAAAAACTTCGAGCTACTAGGCAAGTC I A H P G G P A I L D O V E L K L G L K L E K L R A T R O V TTGAGTGACTATGGAAACATGTCTAGTGCTTGTGTTCTATTCATTTTGGATGAAATGAGAAAGGCCTCAGCCAAAGAAGGACTTAATACT L S D Y G N M S S A C U L F I L D E M R K A S A K E G L N T ACTGGTGAAGGCCTTGATTGGGGTGTGCTTTTCGGATTTGGGCCTGGGCTTACAGTTGAGACTGTTGTACTCCATAGTGTCTCTACTAAT T G E G L D W G U L F G F G P G L T U E T U U L H S U S T N CTCGAGCACCCTAGGGTGCAAGAGAAAGAGTTCGAATAA EHPRVQEKEFE L

Figure 2. The complete cDNA sequence and amino acid sequence of the protein encoded by CHS (GenBank accession number: JN808444).

hidden Markov models revealed that pepper *CHS* and *F3H* did not contain a potential signal peptide with 0.7 and 0.3% probability, respectively (Bendtsen et al., 2004). Using a hidden Markov model algorithm, a

transmembrane topology prediction made by the TMHMM program showed that pepper *CHS* and *F3H* were not potential membrane proteins (Moller et al., 2001). For subcellular localization analysis, the amino acid

ATGCCACCTTCATCCCTAACGTCTCTGGCGAACGAAACTACCGTTCCAACAAGTTTCATTAGGGATGAACAAGAGCGTTCTAAAGTGGCT M P P S S L T S L A N E T T U P T S F I R D E Q E R S K U A TACAACAACTTCAGTGATGACATTCCAGTCATATCGTTGAAGGATATTGATGAGATTGGAACAAGAGGTGAAATATGTGAAAAAATTGTA Y N N F S D D I P V I S L K D I D E I G T R G E I C E K I U GAGGCATGCGAAGATTGGGGCATTTTCCAGGTAGTTGATCATGGGGTAGACCCACAATTAATCTCACAAATGACAAAATTTGCTAAAGAA E A C E D W G I F O U U D H G U D P O L I S O M T K F A K E TTCTTCGCGTTGCCCTCTGAGGAAAAGCTTCGATTTGACATGTCCGGCGGTAAGAAAGGTGGTTTCATAGTCTCTAGCCATCTTCAGGGT F F A L P S E E K L R F D M S G G K K G G F I V S S H L Q G GAAGTGGTCCAAGATTGGCGTGAAATAGTGACCTATTTCTCATACCCAATTCGAGGCTAGAGATTACTCTAGATGGCCAGACAAACCGCAC E U U Q D W R E I U T Y F S Y Р IRARDYSRWPDKPH GGATGGATAGCTGTAACTGAGAAGTACAGTGAAAAGTTAATGGAGTTGGCTTGCAAATTATTAGAAGTACTATCAGAAGCAATGGGTTTG G W I A V T E K Y S E K L M E L A C K L L E V L S E A M G L GAGAAGGAGGCCTTAACCAAGGCATGTGTGGATATGGACCAAAAAGTTGTCGATTTTTACCCAAAGTGTCCACAGCCTGATCTTACC U U U N F Y P K C P Q P D L T EKEALTKACVDMD 0 К CTTGGGTTGAAAAGGCACACTGATCCTGGAACCATCACCCTCTTGTTACAAGACCAAGTTGGTGGTCTTCAAGCCACTAAAGATAATGGC LGLKRHTDPGTITLLL Q D Q V G G L Q A T K D N G AAAACTTGGATCACGGTTAAGCCCCATTGAAGGCGCCTTTTGTTGTTGATCTTGGTGATCATGGTCATTATTTGAGCAACGGGAGGTTCAAG K T W I T V K P I E G A F U U N L G D H G H Y L S N G R F K AACGCTGATCATCAAGCAGTGGTGAACTCGAATAGCAGCAGATTATCGATAGCCACATTTCAGAATCCAGCACCGGAGGCAATAGTGTAT NADHOAUUNSNSS R L S I A T F O N P A P E A I V Y CCATTAAAAATACGAGAAGGAGGAGGAGGAGGCAGTAATGGATGAGCCCATTACATTTGCAGAAATGTATAGGAGGAAAATGAGTAAGGATCTT PLKIREGEKAUMDEPITFAEMYRRKMSKDL GAGGCTGCTAGATTCAAAAAGCTGGCCAAGGAGCAGCAGATACAAGCTGAAGAGGTTGCCGAAAAGGCCAAGTTGGAATCCATGCCCATT E A A R F K K L A K E O O I O A E E V A E K A K L E S M P I GAGGAATTCCTTGCTTAA EEFLA

Figure 3. The complete cDNA sequence and amino acid sequence of the protein encoded by *F3H* (GenBank accession number: JN808445).

sequences were submitted to the PSORT program, and Reinhardt's method showed that pepper *CHS* and *F3H* were probably located in the cytoplasm with upto 45 and 73.9% probability, respectively (Nakai et al., 1999).

Prediction and analysis of structures and conserved domains of pepper *CHS* and *F3H*

Proteins often contained several domains, each with their own evolutionary origins and functions. Examination using the Conserved Domain Architecture Retrieval Tool of BLAST at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) indicated that pepper *CHS* and *F3H* contained one separated conserved domain-*CHS-like* and PLN03176 superfamily, respectively.

The prediction of secondary structure by SOPMA indicates that the deduced pepper *CHS* protein consisted of 45.02% alpha helix, 15.67% extended strands, 6.72% beta turn and 32.59% random coils and pepper *F3H* protein consisted of 37.81% alpha helix, 15.07% extended strands, 5.48% beta turn and 41.64% random coils.

Homology modeling

To better understand the detailed structures of pepper

CHS and *F3H*, homology modeling of *CHS* and *F3H* were performed to estimate their 3D structures, which were similar to that of the alfalfa *CHS* (1bi5A, Single chain) and *A. thaliana* PLN03176 superfamily (1gp6A, single chain). The 3D structures analysis may provide the basis for further study of the relationship between structure and function of these genes from pepper and other species.

Analysis of sequence identity and evolutionary relationships of pepper *CHS* and *F3H*

The deduced protein sequences of pepper *CHS* and *F3H* were submitted to generate BLAST reciprocal best hits, and similarity comparison revealed that pepper *CHS* protein has high homology with the *CHS* proteins of six other species: *Nicotiana tabacum* (92%), *Rhododendron simsii* (91%), *Petunia* × *hybrida* (91%), *Solanum tuberosum* (90%), *Vitis vinifera* (90%) and *Camellia chekiangoleosa* (92%) (Figure 4). The pepper *F3H* has high homology with the proteins of other eight species: *N. tabacum* (89%), *Petunia* × *hybrida* (88%), *S. tuberosum* (87%), *Solanum lycopersicum* (88%), *Litchi chinensis* (82%), *Actinidia chinensis* (81%), *Citrus* maxima (81%) and *V. vinifera* (82%) (Figure 5).

To evaluate the evolutionary relationships of pepper *CHS* and *F3H* with other species, we constructed a phylogenetic tree using DNAstar, Cluster, Mega and

2393

Nicotiana tabacum Petunia x hybrida	MVTVEEFRRAQCAEGPATVMAIGTATPSNCVDQSTYPDYYFRITNSEHKVELKEKFKRMC MVTVEEYRKAORAEGPATVMAIGTATPTNCVDOSTYPDYYFRITNSEHKTDLKEKFKRMC	60 60
Solanum tuberosum	MVTVE OYRKAORAEGPATILAIGTSTPSNCVDOSTYPDYYFRITNSEHKTELKEKFKRMC	60
Capsicum annuum	MVTVE EVEREORAKGPATIMAIGTATPSNCVDOATYPDYYFRITNSEHMTELKEKFORMC	60
Camellia chekiangoleosa	MVTVE EVERAORAE GPATVMAIGTATPPNCVDOSTYPDYYFRITNSEHKTELKEKFORMC	60
Rhododendron simsii	MUTVE DVRRAORAEGPATVMATGTATESNCVDOSTVPDEVERTTNSEHKAELKEKFORMC	60
Vitis vinifera	MUTUNEURNA OPAEGPA TUMA IGTA TPPNCVDOSTVPDVVER ITNSEHKTELKEKEKRMC	60
		00
Nicotiana tabacum	EKSMIKKRYMHLTEEILKENPNICAYMAPSLDARQDIVVVEVPKLGKEAAQKAIKEWGQP	120
Petunia x hybrida	EKSMIKKRYMHLTEEILKENPSMCEYMAPSLDARQDIVVVEVPKLGKEAAQKAIKEWGQP	120
Solanum tuberosum	DKSMIKKRYMHLTEEILKENPNMCAYMAPSLDARQDIVVVEVPKLGKEAAQKAIKEWGQP	120
Capsicum annuum	DKSMIKKRYMHLTEEILKENPNICEYMAPSLDARQDIVVVEIPKLGKEAAKKAIKEWGQP	120
Camellia chekiangoleosa	DKSMIKKRYMYLTEEILKENPNVCAYMAPSLDARQDMVVVEVPKLGKEAATKAIKEWGQP	120
Rhododendron simsii	DKSMIKKRYMYLTEEILKENPSVCEYMAPSLDARODMVVVEVPKLGKEAATKAIKEWGOP	120
Vitis vinifera	DKSMIKKRYMHLTEEILKENPNVCEYMAASLDARQDMVVVEVPKLGKEAAAKAIKEWGQP	120
Nicotiana tabacum	KSKITHL <mark>V</mark> FCTTSGVDMPGCDYQL <mark>T</mark> KLLGLRPSVKRF <mark>MM</mark> YQQGCFAGGTV <mark>L</mark> RMAKDLAEN	180
Petunia x hybrida	KSKITHLFFCTTSGVDMPGCDYQL <mark>T</mark> KLLGLRPSVKR <mark>L</mark> MMYQQGCFAGGTV <mark>LRL</mark> AKDLAEN	180
Solanum tuberosum	KSKITHL <mark>V</mark> FCTTSGVDMPGCDYQL <mark>A</mark> KLLGLRPSVKR <mark>L</mark> MMYQQGCFAGGTV <mark>LRL</mark> AKDLAEN	180
Capsicum annuum	KSKITHL <mark>V</mark> FCTTSGVDMPG <mark>A</mark> DYQL <mark>T</mark> KLLGLRPSVKR <mark>L</mark> MMYQQGCFAGGTVIR <mark>L</mark> AKDLAEN	180
Camellia chekiangoleosa	KSKITHL <mark>V</mark> FCTTSGVDMPG <mark>A</mark> DYQL <mark>T</mark> KLLGLRPSVKR <mark>L</mark> MMYQQGCFAGGTVLRLAKDLAEN	180
Rhododendron simsii	KSKITHL <mark>V</mark> FCTTSGVDMPG <mark>A</mark> DYQL <mark>T</mark> KLLGLRPSVKR <mark>L</mark> MMYQQGCFAGGTV <mark>LRL</mark> AKDLAEN	180
Vitis vinifera	KSKITHL <mark>V</mark> FCTTSGVDMPG <mark>A</mark> DYQL <mark>T</mark> KLLGLRPSVKRFMMYQQGCFAGGTV <mark>LRL</mark> AKDLAEN	180
		0.40
Nicotiana tabacum	NKGARVLVVCSEITAVTFRGPNDTHLDSLVGQALFGDGAAAVIIGSDFIPEVERPLFELV	240
Petunia x hybrida	NKGARVLVVCSEITAVTFRGPNDTHLDSLVGQALFGDGAGAIIIGSDFIFGVERPLFELV	240
Solanum tuberosum	NKGARVLVVCSETTAVTFRGPSESHLDSLVGQALFGDGAAATIMGSDPTIGVERPLFELV	240
Capsicum annuum	NKGARVLVVCSETTAVTFRGPSDTHLDSMVGQALFGDGAAALIVGSDFLPEVERPLFELV	240
Camellia cheklangoleosa	NKGARVLVVCSEITAVTFRGPSDAHLDSLVGQALFGDGAAAIIVGSDFIFEVEKPLFELV	Z 40
Rhododendron simsii	NKGARVLVVCSEITAVTFRGPSDTHLDSLVGQALFGDGAAAIIVGADPVPEVEKPLFELV	240
vitis vinifera	NKGARVLVVCSEITAVTERGESDTHLDSLVGQALEGDGAAAVIVGSDEIPGVEKEMEELV	240
Nicotiana tabacum	SAACTLEDSEGATOCHLEEVGLTEHLLKDVPGLTSKNTEKSLVEAFOPLGTSDWNSLEW	300
Petunia x hybrida	SAAQTI LODSHGA TOGHL REVGL TEHLI KOVEGL TSKNTEK SLEEAEK PLGTSDWASLEW	300
Solanum tuberosum	SAAQTI VEDSECATOCHI DEVCI TEHLI VOVECI I SKNTEKSI LEAFODI CISDWASI EW	300
Capsicum annuum	SAAQTI PDSEGATDCHIDEVCLTEHLIKDVEGLISKNIEKSLIEAFOPLGISDWNSTEW	300
Camellia chekiangoleosa	SAACTI DOSD CATDONINA YOUT THIS ROVIOLIS AND REAL AND A THE START OF A THE SAACTI	300
Rhododendron simsii	SAAQTIL DDSDCATDCHIDEVCLUFFHLLKDVPCLTSKNTEKALTEAFODLCTSDWNSTEW	300
Vitis vinifera	SAAQTTLEDSDGATDGHLREVGLTFHLLKDVPGLTSKNTEKSLNEAFOPLGTKDWNSTFW	300
		000
Nicotiana tabacum	IAHPGGPAILDOVELKLGLKOEKLKATRKVLSNYGNMSSACVLFILDEMRKASAKEGLGT	360
Petunia x hybrida	IAHPGGPAILDOVEIKLGLKPEKLKATRNVLSDYGNMSSACVLFILDEMRKASAKEGLGT	360
Solanum tuberosum	IAHPGGPAILDOVELKLGLKOEKLRATREVLSNYGNMSSACVLFILDEVRKASTNEGLGT	360
Capsicum annuum	IAHPGGPAILDOVELKLGLKLEKLRATROVLSDYGNMSSACVLFILDEMRKASAKEGLNT	360
Camellia chekiangoleosa	IAHPGGPAILDOVELKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKSSAKEGLKT	360
Rhododendron simsii	IAHPGGPAILDOVELKLSLKPEKLRATRHVLSEYGNMSSACVLFILDEMRRKSAEEGLKT	360
Vitis vinifera	IAHPGGPAILDOVEEKLALKPEKLRSTRHVLSEYGNMSSACVLFILDEMRRKSAEEGLKT	360
	~	
Nicotiana tabacum	TGEGL <mark>E</mark> WGVLFGFGPGLTVETVVLHS <mark>VA</mark> T	389
Petunia x hybrida	TGEGLEWGVLFGFGPGLTVETVVLHSVAT	389
Solanum tuberosum	TGEGL <mark>É</mark> WGVLFGFGPGLTVETVVLHS <mark>VA</mark> T	389
Capsicum annuum	TGEGLDWGVLFGFGPGLTVETVVLHS <mark>V</mark> STNLEHPRVQEKEF	401
Camellia chekiangoleosa	TGEGLEWGVLFGFGPGLTVETVVLHSLST	389
Rhododendron simsii	TGEGLEWGVLFGFGPGLTVETVVLHSLCT	389
Vitis vinifera	TGEGL E WGVLFGFGPGLTVETVVLHS <mark>V</mark> ST	389

Figure 4. Alignment of the protein encoded by the pepper *CHS* and six other types of *CHS* from *N. tabacum* (AAK49457), *C. chekiangoleosa* (ADW11243), *S. tuberosum* (AEN83501), *Petunia x hybrida* (CAA32731), *R. simsii* (CAC88858) and *V. vinifera* (XP_002264019).

DNAman softwares on the basis of the *CHS* and *F3H* amino acid sequences, respectively. This analysis revealed a closer genetic relationship between the pepper *CHS* gene and that of *N. tabacum*, *C. chekiangoleosa*

than with those of *R. simsii*, *V. vinifera S. tuberosum* and *Petunia* × *hybrida* (Figure 6). The pepper F3H gene has a closer genetic relationship with that of *N. tabacum* than with those of *Petunia* × *hybrida*, *S. lycopersicum*, *S.*

Citrus maxima	MAP. STLTALAAEKTIN PSFVRFQDER PKVAYNEFSNEI PVI SLAGIDDVGG KRAEICK	58
Litchi chinensis	MAL. ATLTALAQEKTINASFVRDEDERPKVAYNEFSNEIPVISLAGIDEVDG RRAEICQ	58
Actinidia chinensis	MAPTTTLTALAEEKTLQSKFVRDEDERPKVAYNVFSSEIPVISLAGIDEVDGRRSEICR	59
Vitis vinifera	MAP.TTLTALAGEKTLQSSFVRDEDERPKVAYNDFSNEIPVISLEGIDEVGGRRDEICR	58
Solanum lycopersicum	TSFIRDEEERPKVAYNKFSDEIPVISLOGIDDVNGRRSEICE	42
Solanum tuberosum	MA. STLTALANEKTLOTSFIRDEEERPKVAYNKFSDEIPVISLOGIDDING RRSEICE	57
Capsicum annuum	MPP. SSLTSLANETTVPTSFIRDBOERSKVAYNNFSDDIPVISLKDIDEIGTRGEICE	57
Potunia y hybrida	MAP, STLTALAEEKTLOTSFIRDEDERPKVAYNOFSNEIPIISLEGIDDETGKRAEICD	58
Nicotiana tabaoum	MAP, STLTALAFEKTLOTSFTRDEDER PKVAYNOFSDET PUT SLKGIDDESGINGKEGETCE	61
Nicoliana Labacum		
Citrus maxima	KIVEACEDWGIFQVVDHGVDAKLISDMTRLATEFFALPPEEKLKFDMSGGKKGGFIVSSHLQ	120
litchi chinensis	KIVEACEDWGIFOVIDHGVDTKLISDMTRLAREFFALPPEEKLRFDMSGGKKGGFIVSSHLO	120
Actinidia chinensis	KIVEACEDWGIFOVVDHGVDAKLVGEMTRLARDFFALPPEEKLRFDMSGGKKGGFIVSSHLO	121
Vitis vinifera	KTVRACEDNGT POWNIHGVDSNLTSEMT PLAREFFALPPERKLEFDMSGGKKGGETVSSHLO	120
Solanum lyconersicum	PTVNACEDWCIP QV TOHOVD AOLT SOMT KLAKEFFFLD DEFKLEFDMSCCKKCCFTVSSHLQ	104
Solanum tyborocum	TUDIA CEDECVEOUT DUCADA OLT CEMERIA REPERTATE DE LE DE	110
Conciours en num	KIVINACEDWGVE QVIDHGADAQIII SENT KIAKEFEELEFEDEKIKE DISGGKKGGEI VSSHIQ	110
Capsicum annuum	KIVEACEDWGIFQVVDHGVDPQLISQMTKFAKEFFALPSEEKLKFDMSGGKKGGFIVSSHLQ	120
Petunia x ny brida	KIVKACEDWGVFQVVDHGVDAELISQMTTFAKEFFALPPEKKLRFDMSGGKKGGFIVSSHLQ	120
Nicotiana tabacum	KIVKACEDWGIFQVVDHGVDAQLISQMTTLAKQFFALPPEEKLRFDMSGGKKGGFIVSSHLQ	123
		100
Citrus maxima	GEVVKDWREIVTYFSYPKQSRDYSRWPDKP <mark>E</mark> GWMEVTKEYSDQLMGVACKLLEVLSEAMGLE	182
Litchi chinensis	GEAVQDWREIVTYFSYPMRTRDYSRWPDKPQGWIDVTKEYSDKLMGLACKLLEVLSEAMGLE	182
Actinidia chinensis	GEAVQDWREIVTYFSYPIRARDYSRWPDKPDGWRAVTQAYSENLMGLACKLLEVLSEAMGLE	183
Vitis vinifera	GEAVQDWREIVTYFSYPLRTRDYSRWPDKPEGWRSVTQEYSEKLMGLACKLLEVLSEAMDLD	182
Solanum lycopersicum	GEVVQDWREIVTYFSYPIRARDYSRWPDKPQGWIGVTEQYSEKLMDLACKLLEVLSEAMGLE	166
Solanum tuberosum	GEVVQDWREIVTYFSYPIRARDYSRWPDKPQGWIAVTEKYSEKLMDLACKLLEVLSEAMGLE	181
Capsicum annuum	GEVVQDWREIVTYFSYPIRARDYSRWPDKPHGWIAVTEKYSEKLMELACKLLEVLSEAMGLE	181
Petunia x hybrida	GEVVQDWREIVTYFSYPT <mark>RA</mark> RDYSRWPDKP <mark>E</mark> GWIAVTQKYSEKLMELACKLLDVLSEAMGLE	182
Nicotiana tabacum	GEVVQDWREIVTYFSYPIRARDYSRWPDKPDGWIGVTQKYSEKLMELACKLLEVLSEAMGLE	185
Citrus maxima	KEALTKACVDMDQKIVVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDNGKTW	244
Litchi chinensis	KEALTNACVDMDQKVVVNYYPKCPQSDLTLGLKRHTDPGTITLLLQDQVGGLQATRDNGKTW	244
Actinidia chinensis	KEALTKACIDMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDGGKTW	245
Vitis vinifera	KDALTNAC <mark>V</mark> DMDQK V VVN F YPQCP <mark>QP</mark> DLTLGLKRHTDPGTITLLLQDQVGGLQATRDGGKTW	244
Solanum lycopersicum	KEALTKACVDMDQKVVVNFYPKCPEPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDNGKTW	228
Solanum tuberosum	KEALTKACVDMDQKVVVNFYPKCPEPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDNGKTW	243
Capsicum annuum	KEALTKACVDMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDNGKTW	243
Petunia x hybrida	KEALTKACVDMDOKVVVNFYPKCPEPDLTLGLKRHTDPGTITLLLODOVGGLOATKDNGKTW	244
Nicotiana tabacum	KEALTKACVDMDOKVVVNFYPKCPOPDLTLGLKRHTDPGTITLLLODOVGGLOATKDNGKTW	247
Citrus maxima	ITVQPIEGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNSSRLSIATFQNPAPEATVYPLKIR	306
Litchi chinensis	ITVQPVDGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNCSRLSIATFQNPAPEATVYPLKIR	306
Actinidia chinensis	ITVQPVDGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNCSRLSIATFQNPAPEATVYPLAIQ	307
Vitis vinifera	ITVQPVEGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNHSRLSIATFQNPAPEATVYPLKIR	306
Solanum lycopersicum	ITVOPVE GAEVVNLGDHGHYLSNGREKNADHOAVVNSNSSRLSIATFON PAPDAKVYPLKIR	290
Solanum tuberosum	ITVOPVEGAEVVNLGDHGHYLSNGREKNADHOAVVNSNSSRLSIATEONPAPDAKVYPLKIR	305
Capsicum annuum	T TVK PTR GAF VONLODH GHYLSNOR FKN ADHOAVON SNS SRLST AT FON PAPEAT VY PLKTR	305
Petunia x hybrida	TTVO DVE GAEVONI. GDHCHEL SNORFKNA DHOAVONS SSELSTATE ON DA DEAT VY DLKTR	306
Nicotiana tabacum	TTYODVE CAEVANL CDHCHELSNORFKNADHOAVANSNS SKADTATE OVEREBAT VEEKTR	300
	TIVE TRACT THEOREM BUIGHT BUIGHT HUNDER BUILT TERMIN	505
Citrus maxima	EGEK PVLEEPI PESEMYRRKMSKDLELARLKKLANEKHO.DSEKAKLDTKPIEEIL	361
Litchi chinensis	EGEKPILEOPITFAEMYRRKMSKDLELARLKKLAKEOOOODTEKSKLEAKPIEEIL	362
Actinidia chinensis	EGEK PVLEEPT TEAEMYREKMSKDLELARLKKLAKETO, LORORLEKAKLGAKGVEETE	365
Vitis vinifera	EGEKAVLEGET TEAEMYREKMSKOLELARLEKELAKEGO, LOD. VEKAKLESKOT DOTT	362
Solanum lyconersicum	RCRESTMDRPT TRADMYRRKMSKDLELARLKKLAKERKT OTERA KLESK DT PPT L	345
Solanum tuberosum	RCREATMORDT TRAEMY RREATER ARTICLAR OT FRA KIRCHDT PTT	357
Capsicum annuum	RCRKANMPROTURAEMYRRKMSKOLEAAREKKLAKROOTOAREVARKAKLEMITERET	36/
Petunia v hybrida	POPULATING A DIVERSION OF A DIVERSIONO OF A DIVERSIONO OF A DIVERSIONO OF A DIVER	24
Nicotiana tabacum	DODESTINGET TEADILERANDERMONDERANDERANDERANDERANDERANDERANDERANDERA	303
I THE SECOND SEC	BOSKA WIDDELTTABIL KAKISADDELAKEKAKEKAKENUTUAEKAAEKAKEKT KELESTE	200

Figure 5. Alignment of the protein encoded by the pepper *F3H* and eight other types of *F3H* from *Petunia x hybrida* (AAC49929), *S. tuberosum* (AAM48289), *A. chinensis* (ACL54955), *C. maxima* (ADB92595), *L. chinensis* (ADO95201), *S. lycopersicum* (AEK99074), *N. tabacum* (BAF96938) and *V. vinifera* (XP_002267640).



Figure 6. Phylogenetic tree for pepper CHS and F3H genes. a, CHS; b, F3H.

tuberosum, C. maxima, L. chinensis, A. chinensis and V. vinifera (Figure 6).

mRNA tissue-specific expression profile

Semi-guantitative RT-PCR was performed in seven pepper tissues to determine relative expression levels of pepper CHS and F3H mRNA. The continuously expressed gene (Actin), served as an endogenous reference for determination of targeted mRNA profiles. Results reveal that pepper CHS gene was over-expressed in pericarp, moderately in stem and flower, weakly in leaf and placenta, and hardly expressed in root and seed. The pepper F3H gene was over-expressed in pericarp; weakly in leaf, flower and seed, and hardly expressed in root, stem and placenta (Figure 7).

DISCUSSION

Comparative genomics determines the relationship of genome structure and function of different species (Hardison, 2003). Several researchers have shown that

CHS or F3H proteins from different species are highly conserved (Suh et al., 2000; Davies, 1993; Charrier et al., 1995; Gong et al., 1997; Deboo et al., 1995). At the same time, these genes have been characterized at the genetic, chemical and enzymological levels (Ferrer et al., 1999; Jez and Noel, 2000; Niesbach-Klosgen et al., 1987; Koes et al., 1989; Pelletier and Winkel-Shirley, 1996; Shen et al., 2006; Meldgaard, 1992), However, cloning of CHS and F3H genes from pepper has not yet been reported. In this study, pepper CHS and F3H genes were isolated and characterized. The isolated CHS cDNA is 1209 bp long and encodes 402 amino acids. Our comparison of its amino acid sequence showed high homologies (90%) to N. tabacum, R. simsii, Petunia × hybrida, S. tuberosum, V. vinifera and C. chekiangoleosa. The F3H cDNA is 1098 bp long, encodes 365 amino acids and has high homology (more than 81%) with the proteins of eight species: N. Petunia x hybrida, S. tuberosum, S. tabacum, Ivcopersicum, L. chinensis, A. chinensis, C. maxima and V. vinifera. Our results are in accordance with the results with A. thaliana (Pelletier and Winkel-Shirley, 1996) and Pueraria Lobata (Suh et al., 2000). This indicates that genes on the pathway at the primary steps controlling the secondary metabolites of anthocyanin biosynthetic



Figure 7. Tissue expression profile of pepper *CHS* and *F3H* genes. 1, root; 2, stem; 3, leaf; 4, flower; 5, pericarp; 6, placenta; 7, seed. The *Actin* expression level is used for the internal control. M, DL2000 DNA marker; a, *CHS*; b, *F3H*; c, *Actin*.

pathway are very conservative during the plant evolution. Gene expression shows that CHS and F3H genes are specificity expressed with tissue (mostly in photoautotrophic cells) (Todd and Vodkin, 1996). Our results suggest that CHS and F3H were obviously differentially expressed in various tissues (over-expressed in pericarp, moderately or weakly in leaf and flower). The plant of Yunnan Purple Pepper No.1 is violet black, especially the fruit. The CHS and F3H proteins that are accumulated in pericarp, leaf and flower are in accordance with anthocyanin accumulation in these tissues, but the reason why F3H was not checked in stem is unclear. CHS expressed in seed and F3H expressed in placenta may have other function(s).

In summary, we firstly isolated pepper *CHS* and *F3H* genes and performed some necessary analysis. Our results will be extremely important in elucidating the molecular mechanism of anthocyanidins biosynthesis.

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