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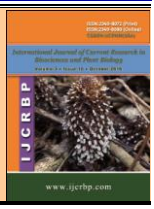

Original Research Article

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Cloning and Sequence Analysis of a Chalcone Synthase (CHS) Gene Involved in Anthocyanin Biosynthesis in *Prunus persica*

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Abstract

Flavonoids are a kind of important secondary metabolites in plants. Usually, it was found in fruits, vegetables, beans, tea and many other plants as combination (flavonoid glycosides) or free states (flavonoid glycosides) form. It has important role in regulating plant growth and development. Chalcone synthase (CHS, EC 2.3.1.74), the first key synthase during the process of flavonoids synthesis, plays an important role in plant growth and development. Based on the whole peach genome sequence, the full length cDNA sequence of chalcone synthase gene (CHS) from *Prunus persica* was isolated by RT-PCR technology. This sequence was 1254 bp, and it included an open reading frame of 1032 bp corresponding to 344 amino acids, and the molecular weight was 37.75 kDa, with the isoelectric point of 5.43, which was designated as *PpCHS* (GenBank accession No. KX823936). Bioinformatics analysis showed that the deduced PpCHS, which shared more than 79% identities with the CHS protein from other plants, was predicted to possess the conserved CHS_like domain, active sites and signal sequences. In the evolutionary tree could be seen the *Fragaria vesca* subsp. *vesca* and *Morus notabilis* genetic relationship is close. The result implied the properties of CHS in the *Prunus persica* was almost as the same as them of other plants. These results indicate that PpCHS belongs to CHS families and provide the basis of research the enzymes and their genes in further step.

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Keywords

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Chalcone synthase
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Introduction

Prunus persica is a deciduous tree native to the region of Northwest China between the Tarim Basin and the north slopes of the Kunlun mountain, where it was first domesticated and cultivated (Faust et al., 1995). It bears an edible juicy fruit called a peach or a nectarine. Some 110 chemical compounds contribute to peach aroma, including alcohols, ketones, aldehydes, esters, polyphenols and terpenoids (Sánchez et al., 2012). Surface color changes of the peach fruit was one of the important indexes for the evaluation of the appearance quality of peach fruit, corresponding color conditions will have a direct influence in their commercial value. The research on the anthocyanin accumulation in the peach fruit generally includes bagging and hormone spraying, etc., but studies on the molecular mechanism of peach fruit color formation are relatively little at present.

Flavonoids are secondary metabolites of plants, which play a decisive role in flower colors, and are suggested to be antioxidant, enzyme inhibitor, plant growth regulator and antitoxin in plants. In addition, flavonoid is one of

the most important components of traditional Chinese medicinal plant, having antioxidant, improvement of human immunity, anti-cancer and other effects on the human body (De León et al., 2007; Courtney-Gutterson et al., 1994). Chemical compounds such as anthocyanin, proanthocyanidins and phlobaphenes are the principal components of plant pigments, which are produced through flavonoid biosynthesis pathway in plants (Winkel-Shirley et al., 2001).

Chalcone synthase (CHS) is the first key enzyme within the synthesis pathway of flavonoids, which is also the first key enzyme in the biosynthesis pathway of peach fruit anthocyanin (Dao et al., 2011). It is responsible for the catalysis of the first step of such pathway, namely, resulting in the catalytic condensation generation of chalcone from termolecular malonyl-CoA and unimolecular 4-coumaroyl-CoA (Hashimoto et al., 1995). Following the isomerization of chalcone, final biosynthesis of anthocyanins is achieved under the action of the enzyme, such as: chalcone isomerase, flavanone 3*β*-hydroxylase, dihydroflavonol-4-reductase, anthocyanins synthase, and UDP-Glc: flavonoid-3-*O*-glucosyltransferase. The pathway of flavonoid in peach leading to anthocyanin synthesis is given in Fig. 1.

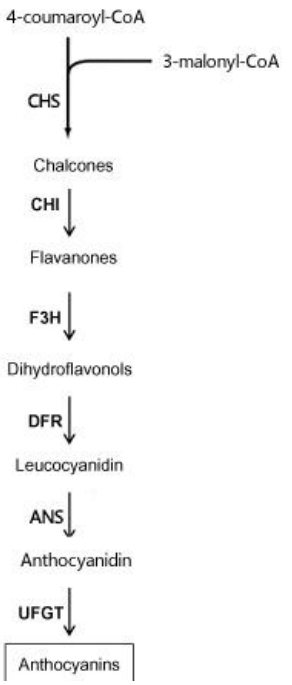

Fig. 1: Schematic diagram of the flavonoid pathway in peach leading to the synthesis of anthocyanins. The enzymes are shown in capital letters and are: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3*β*-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanins synthase; UFGT, UDP-Glc:flavonoid-3-*O*-glucosyltransferase.

Since the publication of the first CHS sequence of parsley in 1972 (Kreuzaler et al., 1972), scientists have cloned the CHS gene sequence from a variety of plants, such as *Arabidopsis thaliana* (Saslowsky et al., 2000), *Glycine max* (Linn.) *M*err (Kurauchi et al., 2011); *Medicago sativa* L. (McKhann et al., 1994), *Pinus* (Schröder et al., 1998), *Petunia hybrida* Vilm (Holton et al., 1993), *Physcomitrella patens* (Koduri et al., 2010), *Cymbidium* ssp. (Liew et al., 1998), *Hordeum vulgare* L. (Rohde et al., 1991), and *Antirrhinum majus* L. (Sommer et al., 1986). But so far, there has not been a report on the complete CHS sequence and expression analysis of peach peel, up till the present moment. Furthermore, many researches relevant to CHS gene have focused on this field since flavonoids are closely related to plant flowers and fruit pigmentation.

In the present study, on the basis of whole genome information of peach trees, RT-PCR technology was applied to clone the full-length cDNA of *PpCHS* sequence from the peach peel for the first time, meanwhile, corresponding open reading frame, homology and phylogenetic trees were further analyzed by bioinformatics tools including DNAMAN, BlAST, and MEGA, so as to construct the expression vector of peach plants in the next step, and to provide basic data for studying the mechanism of expression and regulation of this gene.

Materials and methods

Plant materials

The peach was grown in the horticultural farm of Yangtze University, China. DNA and RNA extraction were collected from peach peels, immediately put them in the liquid nitrogen, and then were stored at Ultra-Low Temperature Freezer type (DW-86L626, Haier Co. Ltd., China) until use.

DNA and RNA extraction

Fruit peel of peach weighing 2g were immediately ground to powder in liquid nitrogen, filtered and after that centrifuged at 10, 000rpm for 10 min. Total RNA was extracted from the pericarps of peach tissues using MiniBEST Plant RNA Extraction kit (TaKaRa, China). Genomic DNA of the peach peel was extracted by the MiniBEST Bacterial Genomic DNA Extraction Kit Ver.2.0 (TaKaRa, China). The purity, concentration, and quality of the total RNA and DNA were tested by 1% (w/v) agarose gel electrophoresis before using.

cDNA synthesis

First-strand cDNA of peach was synthesised with using cDNA Prime Script® 1st Strand cDNA Synthesis Kit (TaKaRa, China). Reaction system contained Oligo dT Primer 2μl, dNTP mixture 2μl, Total RNA 16μl; 5×Primscript buffer 8μl, RNase inhibitor 1μl, Prime script RTase2μl, RNA free H_2O 9μl; under the following conditions: 45°C for 30 min, followed by 75°C for 20 min.

Isolation of the full-length cDNA of PpCHS

The primers CHS-FP (5'-ACTACACATATAGACTGCGCATG-3') and CHS-RP (5'-GATATAGGCTTAGACAGCAAGG-3') were designed and synthesized (Sangon, China) based on the EST sequence of the peach CHS gene. The PCR reaction contained 1μl cDNA products, 2.5μl 10×buffer, 2.0μl $MgCl_2$, 0.5μl TaqDNA polymerase, 1.0 μl dNTP mix and 18μl ddH_2O up to 25 μl. The following conditions: and 96°C for 3 min, followed by 33 cycles of amplification at 96°C for 50s, 56°C for 50s, and 72°C for 70s. This was followed by extension for 5min at 72°C. The PCR product was purified, cloned into the pMD20-T vector (TaKaRa, China), and then sequenced.

Bioinformatics and molecular evolution analyses

The obtained nucleotide sequence and deduced amino acid sequence were compared through database search using online bioinformatics tools (NCBI, http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed by using the website (http://multalin.toulouse.inra.fr/multalin/). The software DNAman 5.0 was used to analyze *PpCHS* gene sequence and amino acid composition. PpCHS and other CHS proteins obtained from GenBank were aligned with the software Vector NTI suit 10.0 program. Phylogenetic tree was constructed by were analyzed using BioEdit 5.0 and MEGA 6.0. The reliability of the tree was measured by bootstrap analysis with 100 replicates. Gene Infinity tool (http://www.geneinfinity.org/index.html) was used to deduce protein isoelectric point (pI) and molecular weight (MW).

Results

cDNA cloning of *PpCHS*

Based on the sequences of EST, the cDNA sequence of *PpCHS* was obtained according to a pair of specific primers and total RNA reverse transcription product. The length of the cDNA sequence of *PpCHS* is 1254bp, and the open reading frame is 1032bp encoding 344 amino acids (Fig. 2). The cDNA sequence of *PpCHS* had high similarity with other CHS genes, and the G+C content of *PpCHS* sequence is 45%.

```
1      ACTACACATATAGACTGCGCATGTGGATCTTGTGAAGGGAAATATTCTCTTGCCAAACCT
61     TTTATCTGTATCATTAATCAGGTGAATTTTTTTTGGAACGTATGATCTATAGTAACAATC
121    TAACAACATAACATACTAGATATCTTGATAGAGCATAGTGTCCTATGTAATTTAAAGGCA
181    ACATTATATGGTTTTAGTAATTCTTATATGGCAATTCACAGGCAAGACAACAACAGTCAA
1             M  V  L  V  I  L  I  W  Q  F  T  G  K  T  T  T  V  K
241    AACTAGGTATGTTGTCATGTCAGATGAGATTCTAGAGAAGTACCCAGAGCTTACAACTGA
19       T  R  Y  V  V  M  S  D  E  I  L  E  K  Y  P  E  L  T  T  E
301    AGGCACACCCACTATAAAGCAAAGACTGCATATTTGTAACGAAGCTGTAACACAGATGGC
39       G  T  P  T  I  K  Q  R  L  H  I  C  N  E  A  V  T  Q  M  A
361    AATTGAAGCTTCAGGAGCTTGCATCAAGAACTGGGGGAGACCTATTTCAGATATAACACA
59       I  E  A  S  G  A  C  I  K  N  W  G  R  P  I  S  D  I  T  H
421    CTTGGTCTATGTCTCATCCAGTGAAGCTCGACTACCCGGTGGTGACATTTACCTAGCAAA
79       L  V  Y  V  S  S  S  E  A  R  L  P  G  G  D  I  Y  L  A  K
481    AGGACTTGGCCTCCGTCCCGAGACTCAAAGGGTCTTGCTTTACTTCTCAGGCTGCTCGGG
99       G  L  G  L  R  P  E  T  Q  R  V  L  L  Y  F  S  G  C  S  G
541    TGGTGTGGCTGGCCTTCGTGTTGCCAAGGACATTGCTGAGAACAATCCAGGAAGCAGAGT
119      G  V  A  G  L  R  V  A  K  D  I  A  E  N  N  P  G  S  R  V
601    ACTACTTGCTACTTCTGAAACCACTATTATTGGCTACAAACCACCAAGTGCACATAGACC
139      L  L  A  T  S  E  T  T  I  I  G  Y  K  P  P  S  A  H  R  P
661    GTATGATCTGGTTGGTGTTGCACTATTTGGGGATGGTGCTGGAGCCATGTTGATTGGCTC
159      Y  D  L  V  G  V  A  L  F  G  D  G  A  G  A  M  L  I  G  S
721    AGACCCTGACTTAATCTCTGAAAAGCCTCTGTTTGAGCTTCACACTGCCATACAAGAGTT
179      D  P  D  L  I  S  E  K  P  L  F  E  L  H  T  A  I  Q  E  F
781    CCTGCCAGACACCGAGAAGACCATTGATGGAAGGGTCACAGAAGAGGGGATTAGTTTCAA
199      L  P  D  T  E  K  T  I  D  G  R  V  T  E  E  G  I  S  F  K
841    GCTAGGAAGAGAGCTTCCTCAGATAATTGAAGATCACATTGAAGGGTTCTGTGGGAGGTT
219      L  G  R  E  L  P  Q  I  I  E  D  H  I  E  G  F  C  G  R  L
901    GATGGGAGTTCTTGGATATGATAACAAGGAGTACAATAAGATGTTTTGGGCTGTTCATCC
239      M  G  V  L  G  Y  D  N  K  E  Y  N  K  M  F  W  A  V  H  P
961    AGGAGGTCCTGCAATCTTGAACCGGTTGGAGAAGCGTCTTGATTTGTTCCCAGAGAAGTT
259      G  G  P  A  I  L  N  R  L  E  K  R  L  D  L  F  P  E  K  L
1021   AAATGCCAGCCGACGAGCTCTGACAGATTATGGCAATGCTAGCAGTAATACCATAGTGTA
279      N  A  S  R  R  A  L  T  D  Y  G  N  A  S  S  N  T  I  V  Y
1081   TGTGCTGGAGTACATGATAGAAGAGAGCAAGAAGATCAAGAAGGAACAGCAAGAAGGAGA
299      V  L  E  Y  M  I  E  E  S  K  K  I  K  K  E  Q  Q  E  G  D
1141   TGGTGAATGGGGATTGATACTGGCTTTTGGACCTGGAATTACTTTTGAGGGAATTCTAGC
319      G  E  W  G  L  I  L  A  F  G  P  G  I  T  F  E  G  I  L  A
1201   AAGGAACCTTGCTGTCTAA GCCTATATCATTTGTTCCTTTAAAATTCATTTTTT
339      R  N  L  A  V  *
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Fig. 2: Nucleotide sequence and deduced amino acid sequence of *PpCHS*.

Characterization of the deduced PpCHS protein

Through analysis with the Computer pI/MW, we found the molecular weight was 37.75 kDa and isoelectric point of 5.43. Blastp aligning analysis found the deduced PpCHS protein has CHS_like, region, and conserved FabH and PLN03169 domains, with a cond_enzymes superfamily (Fig. 3). The cloned PpCHS belongs to CHS superfamily. The secondary structures of CHS proteins were predicted by using SOPMA tool. It was found that PpCHS contents extensive alpha helix (Hh) 31.78%, extended strand (Ee) 26.24%, random coil (Cc) 32.36% and beta turn (Tt) 9.62% (Fig. 4). The secondary structures of deduced PpCHS had high similarities with *Silene latifolia*, *Morus notabilis*, and *Brassica napus* CHS proteins (Table 1).

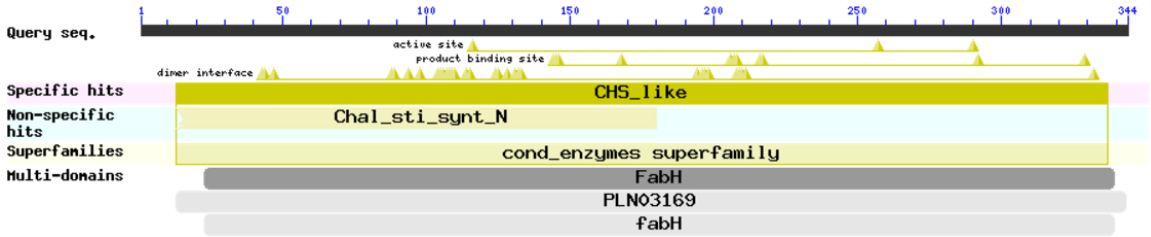

Fig. 3: The amino acid conservative sequence analysis of *PpCHS*. Non-specific hits is the most conserved domain model matching with the query sequence; A superfamily cluster is a set of conserved domain models that generate overlapping annotation on the same protein sequences; Multi-domains are domain models that contain multiple single domains.

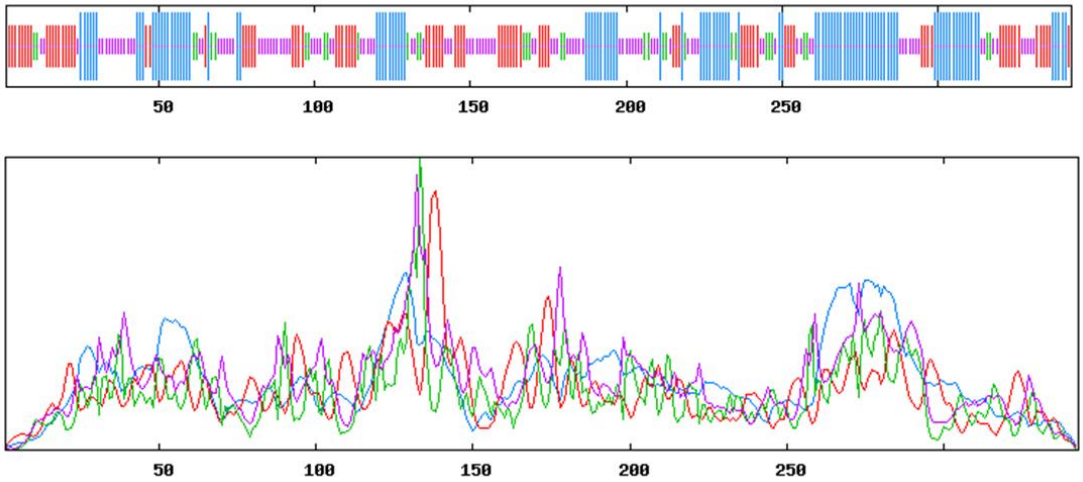

Fig. 4: The predicted secondary structures of PpCHS protein.

Table 1. Secondary structure of CHS proteins (unit: %).

Species	Alpha helix (Hh)	Extended strand (Ee)	Random coil (Cc)	Beta turn (Tt)
Prunus persica	31.78%	26.24%	32.36%	9.62%
Silene latifolia	34.30%	23.22%	32.72%	9.76%
Morus notabilis	38.68%	19.34%	30.03%	11.96%
Brassica napus	36.22%	20.92%	30.87%	11.99%

Multiple alignments of PpCHS protein

Using BLAST search of GeneBank and Vector NTI, as shown in Fig. 5, the PpCHS protein had higher homology with other CHS proteins. The amino acid sequences multiple sequence alignment showed that PpCHS had high identities with *Glycine soja* CHS (85% identities, KHN32314.1), *Cajanus cajan* CHS (84% identities, KYP68086.1), *Morus notabilis* CHS (83% identities, EXB36843.1), *Arabidopsis thaliana* CHS (80% identities, ABK28665.1), *Brassica napus* CHS (80% identities, CDY58814.1), *Silene latifolia* CHS (80% identities, BAE80096.1) and *Cynara cardunculus* var. *scolymus* CHS (79% identities, KVH96005.1). The above analysis shows that PpCHS was a member of the CHS family.

Phylogenetic tree analysis of PpCHS

To investigate the evolutionary relationships in PpCHS and CHSs from other plant species, a phylogenetic tree was constructed by using MEGA 6.0. As shown in Fig. 6, phylogenetic analysis of CHS showed that PpCHS have a closer relationship to *Fragaria vesca* subsp. *vesca* CHS protein than to other CHS proteins. The results showed that the PpCHS protein together with *Glycine soja*, *Cajanus cajan*, *Arabidopsis thaliana*, and *Brassica napus* were grouped into a functional cluster. The above results showed that PpCHS belonged to CHS gene family.

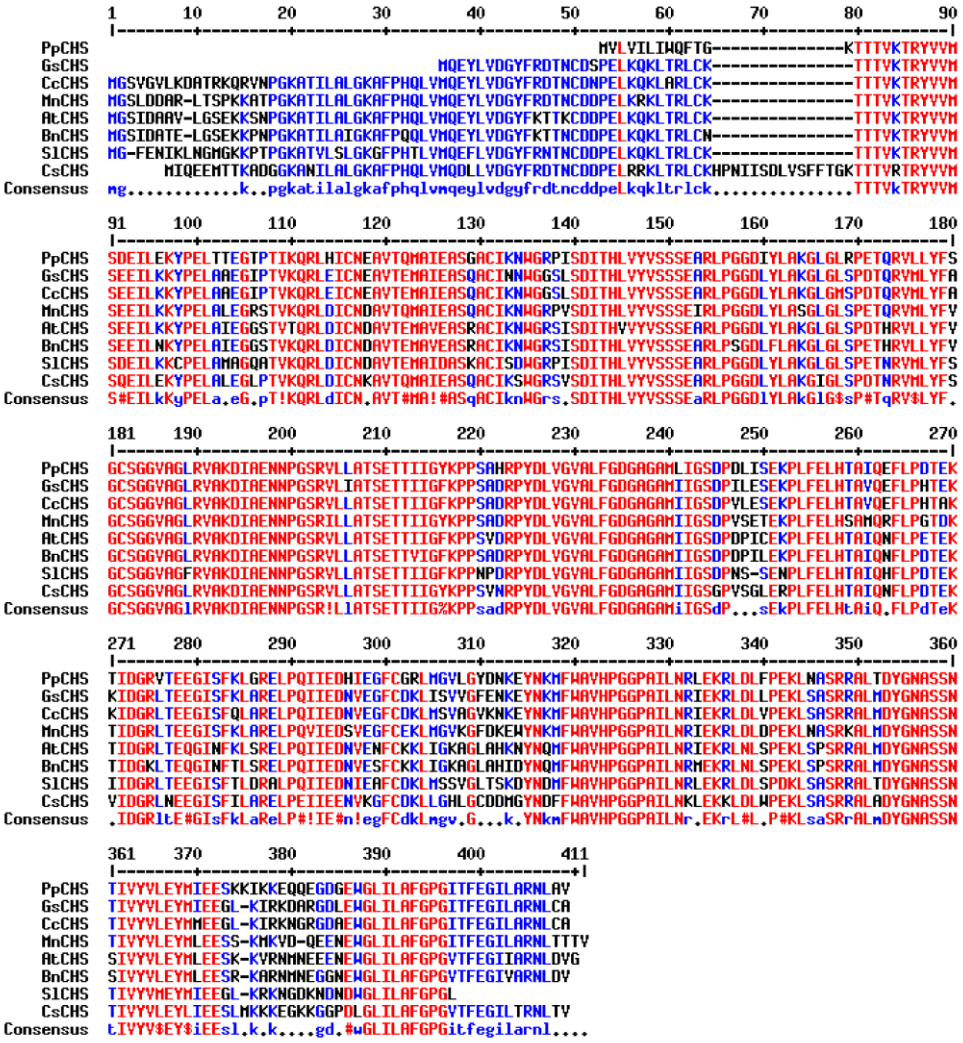

Fig. 5: Sequence multialignment of the deduced PpCHS protein with other plant chalcone synthases. Alignment of some plant Chalcone synthase sequences available on GenBank. *Prunus persica*, PpCHS; *Glycine soja*, GsCHS, KHN32314.1; *Cajanus cajan*, CcCHS, KYP68086.1; *Morus notabilis*, MnCHS , EXB36843.1; *Arabidopsis thaliana*, AtCHS, ABK28665.1; *Brassica napus*, BnCHS, CDY58814.1; *Cynara cardunculus* var. *scolymus*, CsCHS, KVH96005.1; *Silene latifolia*, CsCHS, KVH96005.1; *Silene latifolia*, SlCHS, BAE80096.1.

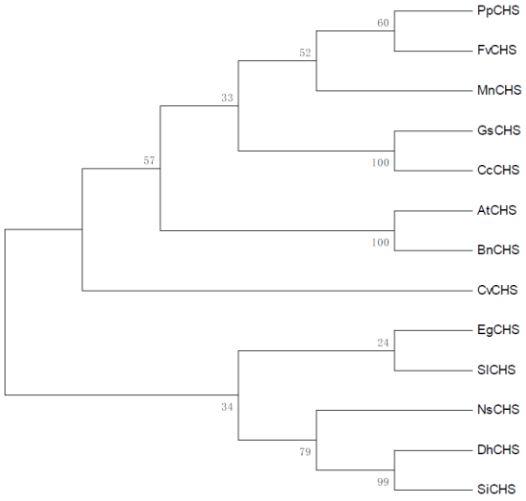

Fig. 6: Phylogenetic analysis of plant secondary product chalcone synthases. The number for each interior branch is the percent bootstraps value (1000 replicates). GenBank accession number are as follows: *Prunus persica*, PpCHS; *Fragaria vesca* subsp. *vesca*, FvCHS, XP_004289833.1; *Dorcoceras hygrometricum*, DhCHS, KZV22002.1; *Glycine soja*,GsCHS, KHN32314.1; *Cajanus cajan*, CcCHS, KYP68086.1; *Morus notabilis*, MnCHS, EXB36843.1; *Eucalyptus grandis*, EgCHS, XP_010028685.1; *Nicotiana sylvestris*, NsCHS, XP_009799363.1; *Sesamum indicum*, SiCHS, XP_011092869.1; *Arabidopsis thaliana*; AtCHS, ABK28665.1; *Brassica napus* BnCHS, CDY58814.1; *Cynara cardunculus* var. *scolymus*, CvCHS, KVH96005.1; *Silene latifolia*, SlCHS, BAE80096.1.

Discussion

Chalcone synthase is the first enzyme in biosynthesis pathway of anthocyanin, the catalytic formation of chalcone is a precursor of various pigments in the metabolic pathways of anthocyanin, corresponding activity directly affects the downstream reaction, and ultimately produces a significant effect on the yield of flavonoids (Shelagh et al., 2001).This study isolated a CHS gene from peach fruit peel by using RT-PCR technique. Results show that the full-length cDNA of *PpCHS* was 1254 bp containing a 1032 bp open reading frame (ORF) encoding 344 amino acids with a calculated molecular mass of 37.75 kDa and an isoelectric point of 5.43. Blastp aligning analysis found the deduced PpCHS protein has CHS_like, region, and conserved FabH and PLN03169 domains, with a cond_enzymes superfamily. The secondary structures of deduced PpCHS had high similarities with *Silene latifolia*, *Morus notabilis*, and *Brassica napus* CHS proteins. The results of nucleic acid homology alignment revealed that the homology of CHS in *Prunus persica* was very high with other plants. It was found that the PpCHS gene encoding for the protein had close relationship with *Fragaria vesca* subsp. *vesca* through phylogenetic analysis. The above results show that the PpCHS protein belonged to CHS proteins family, with synthetase chalcone catalytic function.

Flavonoids are the largest class of secondary metabolites existed in the nature, more than 5000 kinds are known at present, most of them have colors, acting as the main component of plant pigments (Winkel-Shirley et al., 2001). As one of the important secondary metabolic pathways in plants, the regulation system of phenylalanine metabolism pathway is very complex, not only influenced by the control of the body gene, but also affected by the external conditions. Chalcone synthase is a popular element in numerous plants, which plays an important role in the antimicrobial mechanism, stress resistance, cell development and differentiation, the accumulation of pigments and the expression of exogenous genes (Koes et al., 1989).

The activity of chalcone synthase affects the level of the synthesis of flavonoids in plant body, in this regard, the improvement of the expression of plant chalcone synthase gene can promote chalcone synthase activity, which indirectly influence the synthesis of chalcone to provide raw materials for the synthesis of flavonoids. The CHS gene is a super-gene family, the expression patterns of different members are not the same. Plant CHS gene expression is influenced by the stage of plant development and the external environment (Han et al., 2006). Many studies have shown that the CHS gene was actively transcribed when the plant was infected by pathogenic bacteria, wound, UV radiation and exogenous hormones, corresponding activity was markedly increased. Previous research also suggested that the CHS expression was significantly increased after barley, beans, broccoli and so on were infected by pathogenic bacteria (Christensen et al., 1998; Richard et al., 2000).

It has been reported that the yield of the secondary metabolites can be increased by changing the expression of key enzymes in the synthesis pathway of plant secondary metabolites. For example, anthocyanin yield in the sweet wormwood with over expressed CHS gene improved fourfold compared to that in the controls (Chen et al., 1999); The results of the researches showed that following the overexpression of CHS from the yeast in tobacco, anthocyanin yield was obviously improved (Daudonnet et al., 1997). If the expression of CHS gene is increased in peach tree, it is possible to increase the content of anthocyanin in peel and hence improve the quality of fruit. However, currently the transgenic system of peach is not mature, besides, it is difficult to over-express the CHS gene in peach through biotechnology. Even though, based on the in-depth study of the expression and regulation mechanism of *PpCHS*, it is possible to regulate the expression of *PpCHS* by means of hormone or chemical regulation to increase the yield of anthocyanin. On the other hand, this study lays a theoretical foundation for the study of molecular biology on peach peel pigmentation, as well as peach quality improvement and breeding at the molecular level.

Conclusion

PpCHS containing a 1032 bp ORF, encoding 344 amino acids with a calculated molecular mass of 37.75 kDa and an isoelectric point of 5.43 was isolated by RT-PCR from *Prunus persica*. The amino acids of PpCHS protein has CHS_like region, and conserved FabH and PLN03169 domains, with a cond_enzymes superfamily. The phylogenetic tree analysis demonstrated the PpCHS protein was grouped with *Fragaria vesca* subsp. *vesca*. The above results suggest that the *PpCHS* is likely to participate in regulating the anthocyanin biosynthetic pathway of flower and fruit in *Prunus persica*.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

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