Functional study of CHS gene family members in citrus revealed a novel CHS gene affecting the production of flavonoids

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Abstract

Background: Citrus flavonoids are considered as the important secondary metabolites because of their biological and pharmacological activities. Chalcone synthase (CHS) is a key enzyme that catalyses the first committed step in the flavonoid biosynthetic pathway. CHS genes have been isolated and characterized in many plants. Previous studies indicated that CHS is a gene superfamily. In citrus, the number of CHS members and their contribution to the production of flavonoids remains a mystery. In our previous study, the copies of CitCHS2 gene were found in different citrus species and the sequences are highly conserved, but the flavonoid content varied significantly among those species.

Results: From seventy-seven CHS and CHS-like gene sequences, ten CHS members were selected as candidates according to the features of their sequences. Among these candidates, expression was detected from only three genes. A predicted CHS sequence was identified as a novel CHS gene. The structure analysis showed that the gene structure of this novel CHS is very similar to other CHS genes. All three CHS genes were highly conserved and had a basic structure that included one intron and two exons, although they had different expression patterns in different tissues and developmental stages. These genes also presented different sensitivities to methyl jasmonate (MeJA) treatment. In transgenic plants, the expression of CHS genes was significantly correlated with the production of flavonoids. The three CHS genes contributed differently to the production of flavonoids.

Conclusion: Our study indicated that CitCHS is a gene superfamily including at least three functional members. The expression levels of the CHS genes are highly correlated to the biosynthesis of flavonoids. The CHS enzyme is dynamically produced from several CHS genes, and the production of total flavonoids is regulated by the overall expression of CHS family genes.

Keywords: Chalcone synthase, Flavonoid, Gene expression, Gene silencing

Background

Flavonoids consist of over 7000 compounds and represent a large class of plant secondary metabolites [1–3]. In addition to being the primary compounds that determine the colour of flowers, fruits and leaves, flavonoids play important roles in protecting plants against damage from pathogens, pests and herbivores [4, 5], conferring resistance to abiotic stresses [6], and transporting plant hormones in diverse signalling pathways [7]. Flavonoids also have multiple benefits for human health [8], such as the prevention of cardiovascular and carcinogenic risks, promotion of antioxidant and anti-inflammatory activity, and protection against coronary heart disease and certain cancers [9–12].

Flavonoids are produced by all citrus species, such as mandarins, sweet or sour oranges, pummelos, grapefruits, limes and lemons [13]. Thus far, more than 60 flavonoid compounds have been identified in citrus. Those flavonoids can be classified into four major types of substances named flavones, flavonols, flavanones, and flavanonols according to their basic structures [14, 15]. Compared with other plant flavonoids, certain citrus flavonoids possess...
much stronger antioxidant activity due to their unique chemical structures [16–18].

The biosynthesis of flavonoids in plants has been well characterized [19, 20]. Although chalcone synthase (CHS) was identified as the first enzyme involved in the flavonoid biosynthesis pathway in 1972 [21], CHS was not reported in citrus until 1989 [22]. CHS controls the first committed step of flavonoid biosynthesis and catalyzes three molecular malonyl CoA and one molecular 4-coumaryl CoA into naringenin chalcone, which is then rapidly converted into naringenin (flavanone) by chalcone isomerase (CHI) and further synthesized into various flavonoids by the downstream enzymes involved in this pathway [23, 24]. Therefore, understanding the function of the CHS gene and its regulatory mechanism is vital to exploring the genetic control of this metabolite pathway.

In many dicots, CHS is encoded by a multigene family [25–27]. Usually, the chalcone synthase gene forms a family of three to twelve members in most of dicots, such as apple (3 members) [28], mulberry (5 members) [29], Populus (6 members) [30], Glycine max (8~9 members) [31, 32], Viola cornuta (10 members) [33], and petunia (12 members) [34]. In turnip, six CHS genes were cloned and identified, although only three were functional. The other three CHS genes were confirmed to be redundant genes [27]. In Valencia orange, two CHS (CitCHS1 and CitCHS2) genes were identified by Southern blotting. The expression of the two CHS genes in relation to the biosynthesis of flavonoids was very different in citrus cell cultures. CitCHS2 was found to strongly regulate the accumulation of flavonoids, but CitCHS1 did not [35]. In our early study, the CDS (Coding Sequence) fragments of the CitCHS2 gene cloned from ten different citrus species demonstrated high identity [36]. The analysis of flavonoid contents revealed significant differences among different species. However, a strong correlation between the expression of the CitCHS2 gene and the accumulation of flavonoids is only present in a few species.

In the current study, seventy-seven CHS or CHS-like genes were studied to explore the structure and expression profile of the CHS gene family in citrus. The function of the CHS genes was verified by both overexpression and gene silencing via transgenic experiments. The CHS gene family and its activity in regulating the biosynthesis of flavonoids in citrus is discussed.

Results
Phylogenetic analysis of the CHS family genes
The CHS gene has been reported to be a member of the PKS (Polyketide synthase) superfamily in plants [37]. A phylogenetic tree of the CHS family genes was constructed using the ClustalW method based on the substitution of amino acid residues of the CHS and CHS-like genes derived from citrus genome sequence databases (NCBI (https://www.ncbi.nlm.nih.gov/gene), Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and Orange Genome Annotation Project (http://citrus.hzau.edu.cn/cgi-bin/orange/search)). The seventy-seven CHS and CHS-like genes were clustered into mainly three groups (Fig. 1). A more divergent structure of subgroups was found in group I. Group III was noticeably distant from group I and II. The genes in group I and II shared a higher similarity than they do with those in group III. The annotation indicated that most genes from group I and II are the non-functional NADPH-dependent codeinone reductase 2-like gene or type III polyketide synthase related genes. A high identity (75.0% to 88.2%, 1: Table S1) among CitCHS1, CitCHS2 and other 20 genes in group III was observed, which suggested that the citrus CHS family may include many members.

Expression profiles of CHS genes with or without MeJA treatment
To identify the functional members of the CHS family, ten candidate genes from each subgroups of group III of the phylogenet tree were selected according to their similarity and structures and used for the gene expression analysis via qPCR with gene-specific primers (Additional file 1: Table S2). The transcripts were only detected from three genes with or without methyl jasmonate (MeJA) treatment. CitCHS1 (CICLE_v10005133m) and CitCHS2 (CICLE_v10015535m) were two of the three genes mentioned above. The third one, CICLE_v1001405m, has not yet been reported in any publication; it is distinct from the other two CHS genes and located in a different subgroup of the phylogenetic tree. This gene was named CitCHS3 in the present study.

The expression of the CHS genes was tissue specific (Fig. 2). These three genes did not express in the root in the absence of the MeJA treatment. CitCHS1 was not detected in the cotyledon and leaf before the MeJA treatment. However, all three CHS genes were expressed in the stem. MeJA induced the expression of all three genes in the root. The results demonstrated that the three CHS genes responded differently to the MeJA treatment. Overall, CitCHS1 showed the greatest response to the MeJA treatment among the three genes. The expression pattern of the three genes in response to MeJA is tissue specific. In the root, MeJA enhanced the expression of all three genes, particularly CitCHS2 and CitCHS3 after the first two MeJA application. CitCHS2 and CitCHS3 present similar expression profiles in the stem, cotyledon and leaf. MeJA suppressed the expression of these two CHS genes at the early stage of treatment but enhanced the expression at the late stage. CitCHS1 showed similar expression pattern as the other
two genes in the stem, although its expression was enhanced in the cotyledon and leaf.

**MeJA treatment induced the production of flavonoids**

To investigate the distribution of total flavonoids in the four tissues, the contents of four main types of flavonoids, i.e., flavones, flavonols, flavanones and flavanones, were analysed in the seedlings. In the roots, the highest contents of each flavonoid component were detected at time T0 before the MeJA treatment. A sharp decrease in flavonoid contents was observed after the MeJA application, and the content reached a very low level at 12 h after spraying (T1) (Fig. 3). The contents of flavonoids did not significantly change from T1 (12 h after treatment) to T5 (60 h after treatment), only showed slight fluctuations; however, the lowest content of each flavonoid appeared at T0 in the leaves. In leaves, MeJA induced the production of flavonoids in the first 12 h after treatment, and this effect gradually declined through T2 until T3. In the cotyledons and stems, the contents of flavonoids had a similar variation tendency as that in the roots, but the level of variation was much less significant.

**Correlation between CitCHS expression and flavonoid production under MeJA treatment**

To identify the function of the three CitCHS genes, the correlation between gene expression and flavonoid accumulation was studied (Table 1). Among the three CHS genes, the expression of CitCHS1 was not positively correlated with the flavonoid content, including the four primary compounds, in most tissues except the roots, which showed a correlation coefficient of 0.77 between the expression level and total flavonoids. The expression level of the other two genes, CitCHS2 and CitCHS3, was positively correlated with individual flavonoid accumulation in the root, cotyledon and stem, but not the leaf. However, the expression level of both CitCHS2 and CitCHS3 was found to be correlated with the accumulation of total flavonoids in the leaf. The highest
correlation for CitCHS2 was found in the stem ($R = 0.81$). CitCHS3 was found to be positively correlated with flavones and flavonols in the root, with flavanones and flavanonols in the cotyledon and stem. Interestingly, the expression of CitCHS3 was more significantly correlated with the content of flavanones and flavanonols and total flavonoids in the root and cotyledon than was that of CitCHS2, although the opposite trend was observed in the stem and leaf. However, the overall expression level of CitCHS was highly correlated with the total flavonoid accumulation in the root, cotyledon, stem and leaf. Moreover, the three CitCHS genes were co-expressed in the root with coefficients of 0.67, 0.64 and 0.60, respectively. In the other three tissues, co-expression was only found between CitCHS2 and CitCHS3.

**Constitution of the CitCHS genes**

CitCHS2 (Accession No. KP720583-KP720592) was cloned from ten different citrus species in our previous work [36]. The cDNAs of CitCHS1 (Accession No. MF784513) and CitCHS3 (Accession No. MF776052) were amplified from grapefruit (Citrus paradisi Macf. cv. Duncan) and ‘Sunred’ (a red-fleshed hybrid of C. clementina Oroval × C. sinensis Moro blood orange), respectively, in this study. The lengths of the CDS of CitCHS1, CitCHS2 and CitCHS3 were 1170 bp, 1176 bp and 1194 bp, respectively. The DNA sequence of the three CHS genes consisted of one intron and two exons, and the first exon was much smaller than the second. Moreover, the length of the first exon for each of the three CHS genes was the same at 180 nucleotides, representing 60 amino acids. The DNA sequence of the three CHS genes showed variation in the second exon. Based on the constructed structure of CHS in alfalfa [37], the sequence of amino acids showed that the three CHSs obtained from citrus plants contained almost all the main features of the CHS model structure (Fig. 4). This analysis indicated that the three citrus CHS genes are the active CHS genes.

**Functional validation of CitCHS genes with transgenic plants**

Virus-induced gene silencing (VIGS) was conducted to validate the function of the three CitCHS genes. Four positive plants were selected from the transgenic plants to analyse the correlation between gene expression and flavonoid production. The non-transgenic plants and plants transformed with the empty vector were used as controls. The three CitCHS genes showed reduced expression in
the plants transformed with an empty vector, at 53.83%, 54.71% and 69.18% of reductions compared with those of the non-transgenic control. However, the reduced gene expression in the empty vector transgenic plants did not result in a significant decrease in the flavonoid content, with only a 3% reduction in total flavonoids. Large differences in the expression of the three CitCHS genes were observed in the non-transgenic control plants. CitCHS2 showed the highest levels of transcripts, whereas CitCHS1 presented a low level. In all four transgenic plants, three CitCHS genes were not completely silenced, although the level of expression was significantly suppressed (Fig. 5).

The average levels of suppression of the three CHS in transgenic plants were 81.03%, 79.67% and 76.60%. Large variations in the level of suppression were observed among the transgenic plants, although the average level of suppression among the three genes was only slightly different from that of the non-transgenic control, which suggested that VIGS has an equal effect on the three CitCHS genes.

The silenced plants produced significantly fewer flavonoids than did the controls. The level of reduction among the four types of flavonoids was different (Fig. 5). Suppressing the expression of the CitCHS genes in the silenced plants resulted in a significant reduction of flavone and flavonol production but had less effect on the production of flavonones and flavanones. However, the total flavonoid production decreased by 41.11% compared with that in the non-transgenic control. The results indicated the importance of CitCHS genes for the production of flavonoids.

The contribution of the three CitCHS genes towards the production of flavonoids is not similar. The reduced CHS gene expression in the empty vector control transgenic plants did not have a lower production of flavonoids. Excluding the empty vector control from analysis, strong correlations of 0.90, 0.43 and 0.80 were observed between the level of gene expression and the total flavonoid content for the three CitCHS genes.

To identify the contribution of CHS genes to the accumulation of flavonoids, four positive overexpression transgenic citrus plants were analysed for both gene expression and flavonoid accumulation. Among the four positive plants, only three (OE-1, OE-3 and OE-4) showed up-regulated CHS expression (Fig. 6). An apparent increase in the production of flavonoids was observed in OE plants. The OE-2 plant showed the lowest level of CHS expression and flavonoid content among the four OE plants. The CHS gene overexpression results also indicated that the CHS genes contributed significantly to the production of flavonoids.

Discussion

The function of the CHS gene in controlling flavonoid biosynthesis has been well-documented in many plant species [22–24]. The CHS superfamily has also been reported in many plants such as soybean [32], turnip [27] and mulberry [29]. Thus far, studies have not discussed the phenomenon of the CHS superfamily or functional members of this family in citrus plants. In this study, the phylogenetic analysis of 77 CHS or CHS-related genes from the citrus genome revealed that the citrus CHS family may include many members. The expression of three CHS members from 10 candidates were identified in different tissues of young seedlings, suggesting that they are active in citrus. One of them appeared as a novel CHS gene and was termed as CitCHS3. Though,
### Table 1

Pearson's correlation coefficients between gene expression and flavonoid accumulation in four tissues of young seedlings based on time point T0-T5

|       | FV + FVL | FN + FNL | TF    | CHS1  | CHS2  | CHS3  |
|-------|----------|----------|-------|-------|-------|-------|
| **Root** |          |          |       |       |       |       |
| CHS1  | −0.18 NS  | −0.5 NS  | 0.77***|       |       |       |
| CHS2  | 0.39***  | 0.19 NS  | 0.48***| 0.67***|       |       |
| CHS3  | 0.52***  | 0.26 NS  | 0.67***| 0.64***| 0.6***|       |
| CHSs  | 0.45***  | 0.2 NS   | 0.59***| 0.75***| 0.96***| 0.79***|
| **Cotyledon** |          |          |       |       |       |       |
| CHS1  | −0.54 NS  | 0.11 NS  | 0.05 NS|       |       |       |
| CHS2  | −0.01 NS  | 0.49***  | 0.52***| −0.37 NS|       |       |
| CHS3  | −0.02 NS  | 0.62***  | 0.65***| 0.03 NS| 0.5***|       |
| CHSs  | −0.31 NS  | 0.58***  | 0.58***| 0.38***| 0.59***| 0.85***|
| **Stem** |          |          |       |       |       |       |
| CHS1  | 0.46***  | 0.29 NS  | 0.34***|       |       |       |
| CHS2  | 0.45***  | 0.45***  | 0.8*** | 0.01 NS|       |       |
| CHS3  | −0.05 NS  | 0.44***  | 0.34***| −0.53 NS| 0.76***|       |
| CHSs  | 0.42***  | 0.86***  | 0.79***| 0.07 NS| 0.99***| 0.75***|
| **Leaf** |          |          |       |       |       |       |
| CHS1  | 0.01 NS   | −0.85 NS | −0.77 NS|       |       |       |
| CHS2  | −0.64 NS  | 0.01 NS  | 0.59***| −0.31 NS|       |       |
| CHS3  | −0.07 NS  | 0.05 NS  | 0.39***| −0.16 NS| 0.78***|       |
| CHSs  | −0.39 NS  | −0.05 NS | 0.48***| −0.16 NS| 0.94***| 0.94***|

**Note:** FV + FVL, Flavones and flavonols; FN + FNL, Flavanones and flavanonols; TF, Total flavonoids; CHSs, total CHS; CHS1, CitCHS1; CHS2, CitCHS2; and CHS3, CitCHS3

NS = Not significantly different at $P < 0.05$; *** = Significant at $P < 0.001$

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**Fig. 4** Structure and activity sites of the three CHS genes obtained from citrus. This figure was drawn according to Austin and Noel [37]. Key sections are highlighted in yellow. The CHS catalytic triad, residues bound to CoA, and other residues important for functional diversity are highlighted in red, green, and blue, respectively. For clarity, only identical residues in the equivalent positions of the aligned sequences are highlighted.
the level of transcripts was detected in another gene, CICLE_v10030398m at T4 in root and at T2 in leaf. We did not carry on the further work to characterize the function of this gene in this study because it only temporarily expressed under the MeJA treatment. Plant hormone, like JA/MeJA (Jasmonate/Methyl jasmonate) can modulate CHS gene expression [38]. It was reported that both ABA and JA are vital signalling molecules in plants as they can induce stress-resistance and participate in the formation of systemic resistance, through the wound signal transduction pathway [39]. Certain chemical elicitors, such as ABA, SA, JA can mimic environmental stress [40], induce the expression of CHS and enhance the activity of CHS in many plants [41–43]. Cross-talk between these chemical signaling pathways is very common in plant responses to abiotic and biotic factors. In the present study, the ‘Sunred’ blood orange hybrid seedlings were treated with MeJA to identify the activity of CHS genes. Of 10 selected CHS and CHS-like genes, the three CHS genes including CHS3 greatly responded to the MeJA treatment. This result confirmed that MeJA could enhance the expression of CHS genes. The correlation analysis showed that there is a tight relationship between flavonoid accumulation and CHS expression in

Fig. 5 Flavonoid contents and 3 CHS expression level in silenced plants **H-1 and H-2 are healthy control plants, wild type. VC-1 and VC-2 are empty vector control plants. VS-1, VS-2, VS-3 and VS-4 are positive silenced plants. CitCHS1, CICLE_v10005133m; CitCHS2, CICLE_v10015535m; CitCHS3, CICLE_v1001405m. Significant differences with the control (all controls) are indicated: * < 0.05; ** < 0.01
the MeJA-treated plants. However, the levels of expression of the three \textit{CHS} genes as well as their correlation with the accumulation of flavonoids were different. The expression of all three \textit{CHS} genes demonstrated significant correlations with the accumulation of total flavonoids, indicating the importance of \textit{CHS} for controlling the biosynthesis of flavonoids in citrus.

In citrus, the \textit{CHS} gene appeared as a large gene family, as reported in other plants. Two \textit{CHS} genes (\textit{CitCHS1} and \textit{CitCHS2}) were reported in previous studies [35]. Three copies of the \textit{CitCHS2} gene were identified in \textit{Citrus sinensis} (L.) Osbeck cv. Ruby. They were located on three different chromosomes [44]. Nine \textit{CHS} genes were studied in ‘Rio Red’ grapefruit (\textit{C. paradisi}) [24]. These \textit{CHS} genes shared 86–87% and 97–99% similarities with \textit{CitCHS1} and \textit{CitCHS2}, respectively. Presumably, these genes should be the copies of different \textit{CitCHS} members. Only a slight difference was observed in the amino acid sequences among most \textit{CHS} genes in citrus, although variations in their activity in the control of flavonoid biosynthesis were observed. Therefore, we attempted to study the functional characteristics of \textit{CHS} members in this study.

Comparison of genomic DNA sequence, the cDNA structure of the \textit{CHS} gene had an intron and two exons with the same length of 60 amino acids in the first exon and shared over 90% sequence similarity, whereas the second exons were much less conserved, which indicated that the first exon is important for the activity of chalcone synthase because it is part of the basic structure of \textit{CHS}. The novel \textit{CHS} gene identified in this study (i.e., \textit{CitCHS3}) shared high identity with the reported \textit{CHS} and demonstrated a close correlation with the production of flavonoids. This novel \textit{CHS} should represent a new member of the \textit{CitCHS} family, suggesting that the \textit{CHS} gene family in citrus contains at least three functional members and each member may have multiple copies.

Among these three functional members, the expression of \textit{CitCHS1} was not induced by embryogenesis in citrus [35]. Similarly, the present study showed that the expression level of \textit{CitCHS1} was low and maintained a relatively steady level in the four studied tissues compared with the other two \textit{CitCHS} genes. Thus, \textit{CitCHS1} is likely a tissue-specific gene or is not sensitive to MeJA treatment. \textit{CitCHS2} is a well-recognized gene. In this
study, the CitCHS3 gene demonstrated a high level of expression. The correlation analysis also showed that the CitCHS3 gene was co-expressed closely with CitCHS2 ($r > 0.83$) and had a high correlation ($r = 0.6$) with the accumulation of total flavonoids in silenced plants (Table 2).

Although the reduction of the CHS expression were found in the empty vector control plants with 3% of flavonoids reduction. The reduction of both the CitCHS3 gene expression and the flavonoids contents are significantly correlated in the silenced plants, in comparison with both non-transgenic control and empty vector control plants. CHS is located at an important regulatory point upstream of the flavonoid biosynthetic pathway. It can channel the flux of the phenylpropanoid pathway towards flavonoid biosynthesis [43]. Thus, up- or down-regulation of CHS gene expression may strongly affect the production of flavonoids. In previous study, PAL (Phenylalanine ammonia-lyase) shared a similar expression pattern with CHI [45], though no consistent rules were found regarding PAL expression or its influence on CHS in pears [46]. We also tried to illuminate the correlation among PAL, CHS and CHI. Our result showed that the overexpression of CHS may positively affect the expression of CHI gene, which is located downstream of CHS, but no obvious influence can be found to PAL gene, which is located upstream of CHS in the flavonoid biosynthesis pathway.

Conclusions
A novel CHS gene named CitCHS3 (Accession No. MF776052) was identified in citrus plants. CHS is a superfamily in the citrus genome with at least three functional genes that can regulate the biosynthesis of flavonoids. Three CitCHS genes have unique spatial and temporal expression properties and contribute differently to the production of flavonoids.

Materials and methods
Plant materials and methyl jasmonate treatment
Seeds were collected from the mature fruits of ‘Sunred’ blood orange hybrid (Citrus clementina Oroval × Citrus sinensis ‘Moro’) in the field of CREC, UF, on Nov. 2016. The seeds were germinated in soil after the removal of both outer and inner seed coat and grown in a greenhouse under a natural light cycle. Four-week-old seedlings were used in this study. JA/MeJA can modulate CHS gene expression [38]. MeJA (Sigma Company, USA) was prepared at a concentration of 200 μM according to the method of Shi [47]. The seedlings were sprayed with MeJA every 12 h immediately after sampling. The control was sprayed with distilled water. The leaf, stem, cotyledon and root samples were collected every 12 h. T0 is the control without any treatment (only water). T1 (12 h) is 12 h after treatment (same convention for T2 (24 h), T3 (36 h), T4 (48 h), and T5 (60 h)). Samples from 10 to 15 seedlings were mixed together, with three replications performed for each time point. The samples were immediately rinsed in distilled water, placed into liquid nitrogen for freezing, and then stored at −80 °C for further use. The samples were ground into a fine powder in liquid nitrogen for both RNA extraction (Agilent Plant RNA Isolation Kit (Agilent, USA)) and flavonoids detection.

Total RNA isolation and cDNA synthesis
Total RNA was extracted according to the protocol of the Agilent Plant RNA Isolation Kit (Agilent, USA). The integrity and concentration of RNA were determined via 2.0% agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo, Waltham, MA, U.S.A.), respectively. One microgram of DNA-free RNA was initiated using a mixed primer (oligo (dT): random primer = 1.7:0.3, V: V, concentration: 10 μM) for first-strand cDNA synthesis with an Affinity Script QPCR cDNA Synthesis Kit (Agilent, USA) following the manufacturer’s instructions. The product was diluted in a 4-fold volume of sterile deionized water and stored at −20 °C.

Expression analysis
The relative expression of ten candidate CHS genes selected from the phylogenetic analysis was evaluated via qRT-PCR with SYBR Green QPCR Master Mix (Agilent, USA). The qPCR analysis was performed with a CFX96TM Real-Time System (Bio-Rad, USA) in a total volume of 20 μL containing 10 μL of 2× SYBR Green QPCR Master Mix (Agilent, USA), 0.1 μM specific primers (each), and 10 ng of cDNA template. The RNA used in this experiment were extracted through Agilent Plant RNA Isolation Kit (Agilent, USA). The reaction mixtures were heated to 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. The differences in gene expression were calculated using the $2^{△△Ct}$ analysis method. The level of transcription was determined by relative quantification using the citrus GAPDH gene as the reference gene [48].
Three different RNA (of three separated biological replicates) isolations and cDNA syntheses were used as replicates for the qRT-PCR.

Isolation of CHS genes

Genomic DNA and total RNA were extracted from the young leaves of citrus plants using the CTAB method. RNA was extracted according to the protocol of the Agilent Plant RNA Isolation Kit (Agilent, USA). Gene specific primers were designed using NCBI online primer-design software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The RT-PCR reactions were conducted through a program of 95 °C for 5 min, 58 °C for 25 s, and 72 °C for 1 min, 35 cycles, at last 5 min time for extension. All the PCR products were purified through the QIAquick Gel Extraction Kit (QIAGEN, US) and cloned into p-EASY vectors (Transgene, China). The positive clones were sent for sequencing in Eton Bioscience Company (US). The acquired sequences were submitted to Genbank.

Flavonoid detection

The method of detecting flavones and flavonols was modified from the aluminium chloride colourimetric method reported by Woisky and Slatino [49] and Chang [50]. The criterion solutions were generated via step by step dilution with standard quercetin and chromatography-grade methanol in consecutive concentrations of 1000 μg/mL, 500 μg/mL, 250 μg/mL, 100 μg/mL, 50 μg/mL and 25 μg/mL. The absorbance was measured at 415 nm with a Benchmark Plus microplate spectrophotometer (Bio-Rad, USA). The standard curve is in Additional file 1: Table S3-A. One gram of powdered sample was extracted twice with methanol. The first extraction was with 10 mL methanol, and the second was with 5 mL methanol. Each extraction was incubated at 50 °C and subjected to shaking at 200 rpm for 30 min. The residual was removed by centrifugation at 10,000 r/min for 10 min. After a final centrifugation at 10,000 r/min for 10 min, the 0.5 mL of methanol extract was reacted with aluminium chloride before measuring the absorbance at 415 nm with the spectrophotometer.
The protocol for detecting flavanones and flavanons was slightly modified from the method described by Chang [30]. Naringenin was used as a standard chemical to generate criterion solutions at concentrations of 50, 100, 200, 500, 1000, 3000, 4000 and 5000 μg/mL with methanol. The absorbance was measured at 495 nm. The standard curve is in Additional file 1: Table S3-B. One gram of the powdered sample was extracted twice with methanol as described above and reacted with 2,4-dinitrophenylhydrazine, and the flavonoid content was determined by measuring the absorbance at 495 nm.

Overexpression of CitCHS2

The complete ORF fragment of the CitCHS2 gene was amplified from citrus cDNA via PCR using a gene-specific primer with added XbaI and SacI sites cloned into a p-EASY vector (Transgene, China) (Fig. 7a and b). After confirmation by sequencing, the constructed vector was digested with the XbaI and SacI enzymes. Moreover, the PBI121 (Clontech Laboratories, USA) vector was also digested with XbaI and SacI (Fig. 7c). The digested fragments were separated on a 2% agarose gel. The ORF fragment of the CitCHS2 gene was fused to the PBI121 vector with T4 DNA ligase (Transgene, China). The plasmid was transformed into a disarmed strain of Agrobacterium tumefaciens, EH105. An empty vector of PBI121 was also transformed into EH105 as a control. The 4-week-old epicotyl of sweet orange (C. sinensis) with the same phenological period was transformed by Agrobacterium infection according to the method described by Horsch et al. [51].

Virus-induced CHS gene silencing

Gene-specific primers were designed from a highly conserved region of the CitCHS2 gene. A 345-nucleotide sequence was amplified for gene silencing. The fragment was fused to a CTV vector (Fig. 8a). The Agrobacterium tumefaciens strain EHA 105 was transformed with the binary plasmid containing CTV, the target gene fragment and silencing suppressors. Nicotiana benthamiana plants were used for infection to maximize the virus titre. Then, one-year-old ‘Pineapple’ orange trees with the same phenological period were used for inoculation infection with virions partially purified from the sap derived from agroinfiltrated N. benthamiana leaves [52]. The distribution of the CTV vector in the leaves of both the lower and upper parts of the plant was confirmed by ELISA according to the protocol of Garnsey [53]. A double antibody sandwich indirect enzyme-linked-immunosorbent assay (DAS-I-ELISA) [53] was used.
with purified IgG from rabbit polyclonal antibody CTV-908 (1 µg/ml) for coating, and a broadly reactive CTV Mab172 was used for detection. Total RNA was extracted from the leaves in the upper part of the inoculated citrus plants, and the synthesis of first-strand cDNA was performed as previously described. The integrity of the cDNA and silencing sequence were confirmed via PCR.

**Correlation analysis**

The correlation analysis was conducted through Pearson’s correlation analysis. Three separated biological replicates in all time points (including T0) were used for analysis.

**Additional files**

*Additional file 1: Table S1. Identify homology of CHS or CHS-like genes considered in Fig. 1. Table S2. Information of the CHS candidate genes and primers used for expression analysis. Table S3. Standard curve used in flavonoids detection. (DOCX 127 kb)*

**Abbreviations**

CDS: Coding Sequence; CHI: Chalcone isomerase; CHS: Chalcone synthase; CoA: Coenzyme A; MeJA: Methyl jasmonate; PAL: Phenylalanine ammonia-lyase; PKS: Polyketide synthase; VIGS: Virus induced gene silencing

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**Availability of data and materials**

The accession numbers of the three Citrus genes can be found in the NCBI. All data analyzed in this study are included in this published article and its supplementary information files.

**Authors’ contributions**

Z-BW, FG and X-CZ designed the experiments; Z-BW, Q-BY, W-XS and C-AM conducted the experiment; Z-BW and C-AM carried out the virus induced gene silencing experiment; Z- Z-BW and W-XS conducted over-expression experiment; Z-BW and Q-BY measured flavonoid contents; Z-BW, Q-BY and W-XS analysed the data; Z-BW, Q-BY and W-XS prepared manuscript; FG and X-CZ supervised the research and revised the manuscript. All authors have read and approved the manuscript for publication.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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