Integrated Data Reveal The Flavonoid Biosynthesis Metabolic Pathway In *Semen Ziziphi Spinosae* Using Transcriptomic And Metabolomic

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Abstract

Semen Ziziphi Spinosae (SZS) is used to treat neurasthenia, insomnia, and anxiety in China. The extract from SZS contains many bioactive components, such as flavonoids, alkaloids, and terpenoids. Among these, the flavonoids are the main bioactive compounds that have been proven to be responsible for the sedative effects. In this study, a total of 13232 differentially expressed genes and 83 flavonoid metabolites were identified at three different growth and development stages (T1, T2, and T3) of SZS using transcriptomic and metabolomic. The main flavonoid metabolic components of SZS were catechin, L-epicatechin, (-)-epigallocatechin, (+)-gallocatechin, spinosin and its derivatives. A total of 53 unigenes encoding 15 enzymes were identified as candidate genes involved in flavonoid biosynthesis in SZS. Among, PAL, C4H, 4CL, CHS, CHI, FLS, FNS, ANS, ANR, LAR, and UFGT genes were all downregulated in T3 than in T1. The expression levels of these genes influence the accumulation of flavonoids in SZS. Our results should provide valuable information for flavonoid metabolites and candidate genes involved in flavonoid biosynthesis on SZS.

Introduction

Semen Ziziphi Spinosae (SZS), the seed of *Ziziphus jujuba* Mill. var. spinosa (Bunge) Huex H.F. Chou, is a traditional Chinese medicine that has been widely used to treat neurasthenia, insomnia, anxiety, and other symptoms in China for a long time[1,2,3]. SZS was recorded as an herb with sedative and hypnotic effects in the *Shennong bencao jing* (medicine monograph of China) 2500 years ago and is chiefly grown in Hebei, Shanxi, Henan, and Shandong provinces in China[4,5]. It has been used to treat depression, anxiety, and insomnia in Asian countries[6,7]. Modern analytical chemistry studies showed that SZS contains many bioactive components, such as flavonoids, alkaloids, and terpenoids[8,9,10]. The flavonoids of SZS are the main bioactive compounds that have been proven to be responsible for its sedative effects[11]. Especially, the SZS spinosin content is higher than 0.08% (w/w); spinosin is considered to be an active flavonoid for improving sleep[12].

Flavonoids are known for their high medicinal value and widely found in medicinal plants, such as *Perilla frutescens*[13], *Salvia miltiorrhiza*[14], and *Dendrobium catenatum*[15]. Flavonoids can be classified into several categories, e.g. flavonoids, flavonols, flavones, flavanones, isoflavones, and anthocyanins. Their basic skeletons include kaempferol, apigenin, and quercetin. Flavonoids biosynthesis is related to phenylpropanoid pathway, flavonoid pathway, isoflavonoid pathway, flavone and flavonol pathway, and anthocyanin pathway. The key enzymes of flavonoids biosynthesis are phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), chalcone synthase (CHS), chalcone isomerase (CHI), favonol synthase (FLS), and flavone synthase (FNS), etc. Wu et al. (2010) first reported 24 unigenes that were identified using transcriptomic technology, which were related to ginsenosides biosynthesis in Panax quinquefolius[16]. In recent years, transcriptomic technology has widely used to mine the key gene in secondary metabolites biosynthesis in plants[17,18].

UPLC/ESI-Q TRAP-MS/MS is used for identification and analysis of plant metabolites and has the advantages of wide coverage, fast separation, and high throughput and sensitivity[19,20]. To date, 36 compounds have been identified in SZS using UPLC-Q/TOF-MS/MS, including 5 nucleosides, 6 amino acids, 2 triterpenoid saponins, 15 flavonoids, 1 lactone, 5 alkaloids, and 2 phenolic acids[21]. Although some bioactive compounds have been identified in SZS, the number and type of flavonoids and the regulating mechanism of flavonoid biosynthesis in SZS are still unclear. In our study, we identified and analyzed the flavonoid metabolites and genes involving the biosynthesis of flavonoid metabolites in SZS using metabolomic and transcriptomic. Our results should provide valuable information for flavonoid metabolites and candidate genes involved in flavonoid biosynthesis on SZS.

Materials And Methods

Plant materials

Ziziphus jujuba spinosa were cultivated in the germplasm resources center of jujube at the Baijiazhuang Village, Taizijing Township, Xingtai City, Hebei Province, China. It is a common species that belongs to the plant family Rhamnaceae. The seed of *Ziziphus jujuba spinosa* (SZS) is oblate or oblong, and the surface is reddish brown or purplish red. We collected SZS from the same tree in the germplasm resources center of jujube every two weeks, on 15 August (T1), 1 September (T2), and 15 September (T3) 2020. We confirm that the collection of plant material is compliant with relevant institutional, national, and international guidelines and legislation. All SZS samples were frozen using liquid nitrogen and stored at -80 °C for RNA and metabolite extraction.

Metabolite extraction

Freeze-dried SZS samples were crushed using zirconia beads and a mixer mill (MM 400, VERDER RETSCH, Shanghai, China). The powder was weighed and 100 mg was resuspended in 1.0 mL 70% aqueous methanol and incubated overnight at 4 °C. The solution (i.e., extract) was centrifuged at 10,000 *g* for 10 min. The supernatant was added to the CNWBOND Carbon-GCB SPE Cartridge (250 mg, 3 mL; ANPEL, Shanghai, China; www.anpel.com.cn/) and then filtered (SCAA-104, 0.22 μm pore size; ANPEL) before UPLC-MS/MS analysis.

Ultra performance liquid chromatography (UPLC) conditions

The SZS sample extracts were analyzed using an UPLC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM30A system, Shanghai, China). The analytical conditions were as follow: UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 mm × 100 mm); solvent system, water (0.04% acetic acid): acetonitrile (0.04% acetic acid); gradient program, 100:0 (v/v) at 0 min, 5:95 (v/v) at 11.0 min, 5:95 (v/v) at 12.0 min, 95:5 (v/v) at 12.1 min, 95:5 (v/v) at 15.0 min; flow rate, 0.40 mL/min; temperature,

40°C; and injection volume: 2 μL. After UPLC, the effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS.

ESI-Q TRAP-MS/MS

The LIT and triple quadrupole (QQQ) scans were acquired using the API 6500 Q TRAP LC-MS/MS system equipped with an ESI Turbo Ion-Spray interface. The system was operated in the positive ion mode and was controlled using the Analyst 1.6 software (AB Sciex). The ESI source operation parameters were as follows: ion source, turbo spray; source temperature, 500 °C; ion spray voltage, 5,500 V; ion source gas I, gas II, and curtain gas were set at 55, 60, and 25.0 psi, respectively; and the collision gas was high. Instrument tuning and mass calibration were performed using 10 and 100 μmol/L polypropylene glycol solutions in the QQQ and LIT modes, respectively. The QQQ scans were acquired in MRM experiments, with the collision gas (nitrogen) set at 5 psi. The declustering potential and collision energy were optimized for individual MRM transitions. A specific set of MRM transitions was monitored for each period according to the metabolites eluted within the period.

Identification and quantitative analysis of metabolites

A publicly available standard metabolite database (Metware Biotechnology Co., Ltd., Wuhan, China), which was built on the basis of multiple ion monitoring–enhanced product ions, was used for the qualitative analysis of metabolites. The quantitative analysis of metabolites was completed using a published multiple reaction monitoring method[37,38]. An unsupervised principal component analysis, hierarchical cluster analysis, and partial least-squares discriminant analysis were performed using the prcomp function within R (www.r-project.org). Metabolites that were significantly differentially abundant between groups were identified according to the following criteria: variable importance in projection value ≥ 1 and an absolute $\log_2$(fold change) ≥ 1.

RNA extraction and Illumina sequencing

Total RNA was extracted from frozen leaves using the RNAprep Pure Plant Kit (TIANGEN Biotech, Beijing, China), after which RNA degradation and contamination were monitored by 1.2% agarose gel electrophoresis. The purified RNA concentrations were quantified using the NanoDrop™ 1000 spectrophotometer (ThermoFisher Scientific, Shanghai, China). Poly (A) mRNA was enriched from the total RNA using Oligo (dT) magnetic beads. The poly (A) mRNA was subsequently fragmented by an RNA fragmentation kit (Ambion, Austin, TX, USA). The fragmented RNA was transcribed into first-strand cDNA using reverse transcriptase and random hexamer primers. Second-strand cDNA was synthesized using DNA polymerase I and RNase H (Invitrogen, Carlsbad, CA, USA). After end repair and the addition of a poly (A) tail, suitable length fragments were isolated and connected to the sequencing adaptors. The fragments were sequenced on the Illumina HiSeq™ 2500 platform[39].

RNA sequencing (RNA-seq) data analysis and annotation

To acquire high-quality reads, the raw reads in fastq format were processed through in-house Perl scripts. Clean reads were obtained from raw data by removing adaptor sequences, low-quality reads, and reads containing ploy-N. All downstream analyses were based on clean data with high quality. Transcriptome assembly was accomplished using Trinity software (version2.5.1)[40]. Genes were functionally annotated according to the Kyoto Encyclopedia of Gene and Genome (KEGG) pathway database (http://www.genome.jp/kegg), the NCBI non-redundant (Nr) database (version 2018.4), the Swiss-Prot protein database (http://www.uniprot.org), the euKaryotic Clusters of Orthologous Groups (KOG) database (version 1.0), the Gene Ontology (GO) database (http://www.geneontology.org), and the Pfam database (version 33.0).

Gene expression levels were estimated by RSEM (version 1.2.26)[41]. Analysis of DEGs of the two groups was performed with the DESeq R package (1.10.1). DESeq provides statistical routines for determining DEGs using a model based on the negative binomial distribution. The results of all statistical tests underwent a Benjamini and Hochberg false discovery rate multiple testing correction. Genes were determined to be significantly differentially expressed at an adjusted P-value < 0.05. The GO enrichment analysis of the DEGs was performed using the topGO R package based on the Kolmogorov-Smirnov test. The pathways significantly associated with the DEGs were identified using the KEGG database (http://www.genome.jp/kegg)[42,43,44] and KOBAS software[45].

QRT-PCR expression analysis of genes involved in flavonoid biosynthesis

Total RNA of SZS was reverse-transcribed using the Quantscript Reverse Transcriptase Kit (TIANGEN Biotech, Beijing, China). The resulting cDNA was used as the template for analyzing gene expression by qRT-PCR. The specific primers involved in the flavonoid biosynthesis genes and actin gene (internal control) are listed in Table S3. The qRT-PCR analysis was performed using SYBR® Premix Ex Taq™ II (TaKaRa; http://www.takarabiomed.com.cn) and the ABI Prism 7500 system, including the software for the 7500 and 7500 Fast Real-Time PCR systems (version 2.0.1) (Applied Biosystems, Foster City, CA, USA). The $2^{-\Delta\Delta CT}$ method was used to quantify gene expression levels[46].

Statistical analysis

Each experiment was three biological replicates. Data were analyzed using Microsoft Office Excel 2013 software. Data were presented as the means ± standard deviations. The significance of any differences was analyzed using $p < 0.05$ as the threshold.

Results

Flavonoid metabolites in SZS

To identify the variation of differential flavonoid metabolites during the growth and development of SZS (Fig. 1), we used a UPLC/ESI-Q TRAP-MS/MS system to detect the flavonoid metabolites in SZS. A total of 83 (T1, 81; T2, 82; T3, 81) flavonoid metabolites were identified in SZS. R software program was used to draw the heatmap of metabolites after unit variance scaling (UV), and the accumulation pattern of metabolites were described using hierarchical cluster analysis (HCA) (Fig. 2A). The 83 flavonoid metabolites were classified into 8 categories, including 3 chalcones, 5 dihydroflavones, 2 dihydroflavonols, 14 flavonols, 9 flavanols, 40 flavonoids, 5 isoflavones, and 5 anthocyanins (Fig. 2B and Table S1).

Differential flavonoids were screened by combining the absolute Log_2(fold change) ≥ 1 and a variable importance in projection value ≥ 1; 35 of 83 flavonoid metabolites were identified as significantly differentially accumulated between T1 and T2 (20 downregulated and 15 upregulated), 32 flavonoids were identified as significantly differentially accumulated between T1 and T3 (24 downregulated and 8 upregulated), and 2 flavonoids were identified as significantly differentially accumulated between T2 and T3 (1 downregulated and 1 upregulated) (Fig. 2C). Analysis of the flavonoid metabolites data indicated that the flavonoid metabolites was higher content in T1 than in T2 or T3. More specially, catechin, L-epicatechin, (-)-epigallocatechin, (+)-gallocatechin were abundant in T1, accounting for more than 50% of the total flavonoid metabolites content, but they were only 5.94% and 4.79% of the total flavonoid metabolites content in T2 and T3, respectively. The content of spinosin and its derivatives, with sedative and hypnotic functions, was not significantly different in T1, T2 and T3 (Table 1).

Table 1
The main flavonoid metabolic components in SZS

Index	Compounds	T1 [a]	T2 [b]	T3 [c]
mws0042	(-)-Epigallocatechin	2.29E + 07	1.25E + 05	1.41E + 05
mws0049	(+)-Gallocatechin	3.20E + 07	2.49E + 05	3.08E + 05
mws0054	Catechin	1.09E + 07	1.62E + 06	8.25E + 05
pme0460	L-Epicatechin	1.45E + 07	1.54E + 06	9.03E + 05
mws1387	Spinosin	4.19E + 04	6.81E + 04	6.88E + 04
pmp000882	6‴-p-Hydroxybenzoylspinosin	2.44E + 05	3.84E + 05	3.56E + 05
pmp000883	6‴-Cinnamoylspinosin	9.61E + 03	7.59E + 03	9.97E + 03
pmp000884	6‴-p-Coumaroylspinosin	1.12E + 06	1.03E + 06	1.02E + 06
pmp000885	6‴-Vanilloylspinosin	9.99E + 05	1.36E + 06	1.48E + 06
pmp000886	6‴-Feruloylspinosin	1.78E + 07	1.75E + 07	1.83E + 07
pmp000887	6‴-Sinapoylspinosin	3.09E + 06	3.21E + 06	3.18E + 06

T1[a], T2[b] and T3[c], the average of three biological replicates.

RNA sequencing and functional annotation

To elucidate the molecular basis of the flavonoid biosynthesis metabolic pathway during the growth and development of SZS, transcriptomes were analyzed to identify the differentially expressed genes (DEGs) in SZS. A total of 61.82 Gb clean data were produced from SZS. The number of clean data of each sample ranged from 6.49 Gb to 7.26 Gb. The Q30 percentages of all samples were more than 91.23%. The GC content of each sample was 44.19% to 45.16%. For functional annotations, all clean data were searched in the KEGG, NR, Swiss-Prot, Trembl, KOG, GO, and Pfam databases. A total of 27,241 genes were annotated functionally according to the BLASTX program, including 25,150 and 24,012 genes annotated according to the GO and KEGG databases, respectively (Table S2).

Based on the GO annotation, 25,150 genes were classified into 58 classifications and involved in 3 major categories (cellular component, molecular function, and biological process) (Fig. 3A). In cellular component category, 17,963 genes and 10,028 genes were annotated with the cell and membrane, respectively. In biological process category, cellular process (14,698 genes) and metabolic process (12,622 genes) were the two most commonly assigned GO terms. In molecular function category, 14,077 and 12,615 genes were annotated with the binding and catalytic activity, respectively. To identify the genes involved in flavonoid biosynthesis, 24,012 genes were classified into 141 metabolism and

biosynthesis pathways in KEGG database. Among, 473 genes were belonged to the phenylpropanoid biosynthesis (ko00940), 148 genes were related to the flavonoid biosynthesis (ko00941), 12 genes contributed to the anthocyanin biosynthesis (ko00942), 63 genes influenced isoflavonoid biosynthesis (ko00943), and 41 genes were involved in flavone and flavonol biosynthesis (ko00944) (Fig. 3B).

Differential expression of genes at three development stages of SZS

To identify the DEGs involved in the development stages of SZS, $|\log_2(\text{fold change})| \geq 1$ and a false discovery rate < 0.05 were used as the filter criteria. In this study, 8725 DEGs were identified in the T1 vs. T2, including 3398 up-DEGs and 5327 down-DEGs. A total of 10925 DEGs were identified in the T1 vs. T3, including 4278 up-DEGs and 6647 down-DEGs. And there are 4086 DEGs identified in the T2 vs. T3, of which 1557 were up-DEGs and 2529 were down-DEGs (Fig. 4A). A total of 13232 unigenes were significantly differentially at three different growth stages of SZS based on the Venn diagram (Fig. 4B). Among these DEGs, 152 CYP450 genes and 115 UDP-glycosyltransferase genes, 238 methyltransferase genes, and 97 acyltransferase genes were detected (Fig. 4C). These DEGs may play an important role in flavonoid biosynthesis of SZS.

Biosynthesis of flavonoid in SZS

We examined the flavonoid biosynthesis pathway in SZS via a combined analysis of the transcriptomic and metabolomic data, and selected T1 and T3 transcriptomic and metabolomic data to carry out correlation test. The results showed that 83 flavonoid metabolites were detected in SZS using UPLC/ESI-Q TRAP-MS/MS system. Most of these flavonoids were more abundant in T1 than in T3. Among, catechin, L-epicatechin, (-)-epigallocatechin, (+)-gallocatechin were more abundant in T1 than in T3, whereas pinocembrin, phloretin, dihydrokaempferol, and pelargonidin were less abundant. Further analysis of the DEGs involved in flavonoid biosynthesis pathway, 53 DEGs that encoded 15 enzymes were mined between T1 and T3, including 7 upregulated and 46 downregulated genes (Table 2). In the present study, we found that PAL, C4H, 4CL, CHS, CHI, FLS, FNS, ANS, ANR, LAR, and UFGT genes were all downregulated in T3 than in T1 (Fig. 5). These downregulated genes affected the biosynthesis of flavonoids in SZS. To verify the accuracy of the transcriptome data, 18 DEGs were selected to validate the sequencing results. The quantitative real-time (qRT)-PCR results indicated that 4 and 14 genes were expressed at higher and lower levels in T3 than T1. Our qRT-PCR results were consistent with the RNA-seq data (Fig. 6).

Table 2

Candidate genes related to flavonoid biosynthesis in SZS

Gene	Enzyme	No.Up	No.Down
PAL	Phenylalanine ammonia-lyase	0	2
C4H	Cinnamic acid 4-hydroxylase	0	2
4CL	4-coumarate CoA ligase	0	4
CHS	Chalcone synthase	0	6
CHI	Chalcone isomerase	0	4
F3H	Flavanone 3-hydroxylase	4	1
FLS	Flavonol synthase	0	7
FNS	Flavone synthase	0	2
F3'H	Flavonoid 3'hydroxylase	1	1
F3'5'H	Flavonoid 3'5'hydroxylase	1	0
DFR	Dihydroflavonol 4-reductase	1	2
ANS	Anthocyanidin synthase	0	2
UFGT	Flavonoid 3-O-glucosyltransferase	0	10
ANR	Anthocyanidin reductase	0	1
LAR	leucoanthocyanidin reductase	0	2

Discussion

SZS is a traditional medicinal herb that has been used in China for centuries. It can treat anxiety, insomnia, and night sweats according to the Chinese Pharmacopoeia[22]. SZS has abundant bioactive secondary metabolites that can help to enhance the body's immunity and treat diseases. Increasing attention has been given to the sedative, hypnotic, and memory effects of SZS[23,24,25]. It was reported that SZS decoction is a classical herbal formula that improves sleep[26]. The mechanism of SZS treatment for insomnia is primarily mediated by the GABAergic and serotonergic systems[27]. Li et al. (2019) reported that the methanol extract of SZS ameliorates ethanol withdrawal (EtOHW) anxiety by improving both CRF/CRFR1 and N/OFQ/NOP transmission in the CeA[28]. An aqueous extract of SZS ameliorates nicotine withdrawal-induced anxiety by improving CRF/CRF1R and noradrenergic signaling in the CeA[29].

In recent years, metabolomics has been utilized to gain insight into the chemical compounds in plants[30,31]. UPLC/ESI-Q TRAP-MS/MS is a good technique to discover secondary metabolites of medicinal plants. In order to elucidate the variation of flavonoid metabolites at different growth stages of SZS, metabolomics analysis was performed using UPLC/ESI-Q TRAP-MS/MS. In our study, we identified 83 differential flavonoid metabolites in SZS (Fig. 2). Further analysis of the flavonoid metabolites

indicated that the main flavonoid components were catechin, L-epicatechin, (-)-epigallocatechin, (+)-gallocatechin in T1, which exceeded 50% of the total flavonoids content, whereas their content reduced rapidly from T1 to T2 or T3. We thought that these compounds could be oxidized or hydrolyzed in the SZS ripening process, which eventually declined the total flavonoid content in T2 and T3. Spinosin, a C-glycoside flavonoid derivative, is one of two main SZS compounds that have been used as a sedative to treat insomnia[32]. In particular, 6‴-feruloylspinosin is involved in the sedative effect in rat hippocampal neurons by enhancing the expression of GABA[33]. In our study, we found that the content of spinosin and its derivatives was not significantly different among the T1, T2, and T3 stages. We speculated that spinosin and its derivatives were mainly found in seed coat rather than seed. Next, we will further detect the contents of these compounds in seed and seed coat, respectively.

Transcriptomic and metabolomic are useful methods to understand the biosynthesis of metabolites in medicinal plants. Gao et al. (2014) combined metabolomic and transcriptomic to analyze the biosynthesis of tanshinone in *Salvia miltiorrhiza*[34]. Li et al. (2020) reconstructed the flavonoid biosynthesis pathway of Mulberry leaves using metabolic profiling and transcriptome, and identified flavonol candidate UDP-rhamnosyltransferases[35]. In our study, transcriptomic analysis of the SZS identified 53 DEGs involving in the flavonoid biosynthesis between T1 and T3 (Fig. 5). Among these DEGs, the expression levels of PAL, C4H, 4CL, CHS, CHI, FLS, FNS, ANS, ANR, LAR, and UFGT genes were all downregulated, which resulted in a low flavonoid content in T3 than T1. ANR is a reductase that converts anthocyanidin into epicatechin. LAR is a key enzyme of the flavonoid pathway that catalyzes leucoanthocyanidin to form catechin. Li et al. (2019) reported ANR and LAR genes that were involved in proanthocyanidin biosynthesis in Malus crabapple, and the expression levels of them were correlated with the contents of catechin and epicatechin[36]. In this study, we detected one ANR and two LAR genes in transcriptomic data of SZS that were all downregulated, which led to the low content of catechin and epicatechin in T3 than T1 (Table S1).

In conclusion, we integrated transcriptomic and metabolomic data to reveal the flavonoid biosynthesis metabolic pathway in Semen Ziziphi Spinosae. A total of 13232 differentially expressed genes and 83 flavonoid metabolites were identified at different growth stages of SZS. Our results greatly enrich the *Semen* Ziziphi Spinosae phytochemical composition database and provide valuable information for further study of the flavonoid biosynthesis on SZS.

Declarations

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Author contributions

T.J. conceived and designed the experiments. L.D., C.X. X.L., and S.Q. performed the experiments. T.J., W.T. analyzed the data. T.J. and W.T. wrote the paper. All authors read and approved the final version of the paper.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Phenotypes of SZS at different growth and development stages.

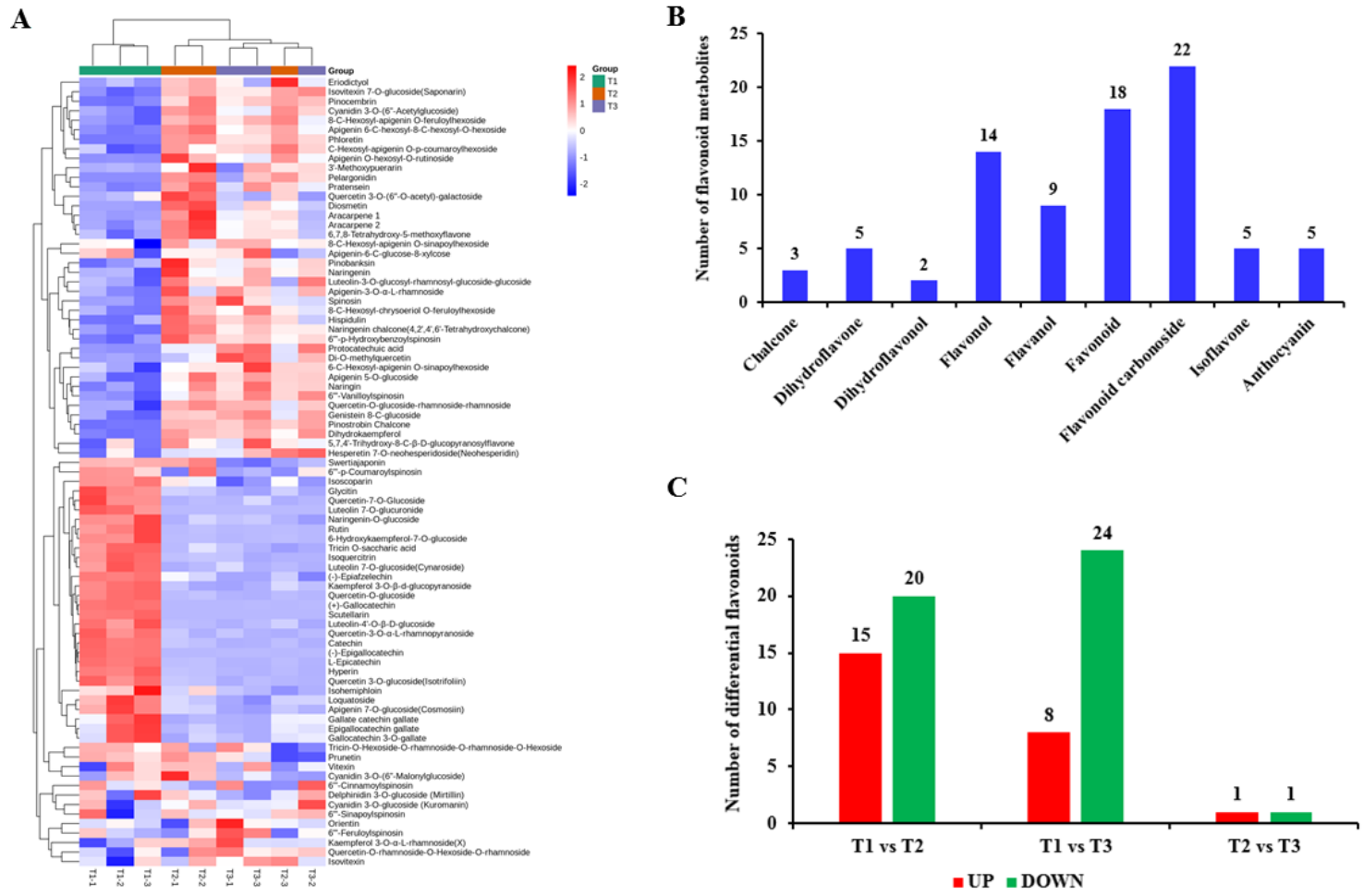

Figure 2

Analysis of flavonoid metabolites in SZS. (A) Cluster heat map of flavonoid metabolites between groups T1, T2, and T3. The abundance of each metabolite is represented with a different color. Red indicates highly abundant metabolites, whereas metabolites with low abundance are shown in blue. (B) The number of flavonoid metabolites in different categories. (C) The number of differential flavonoids between T1, T2, and T3.

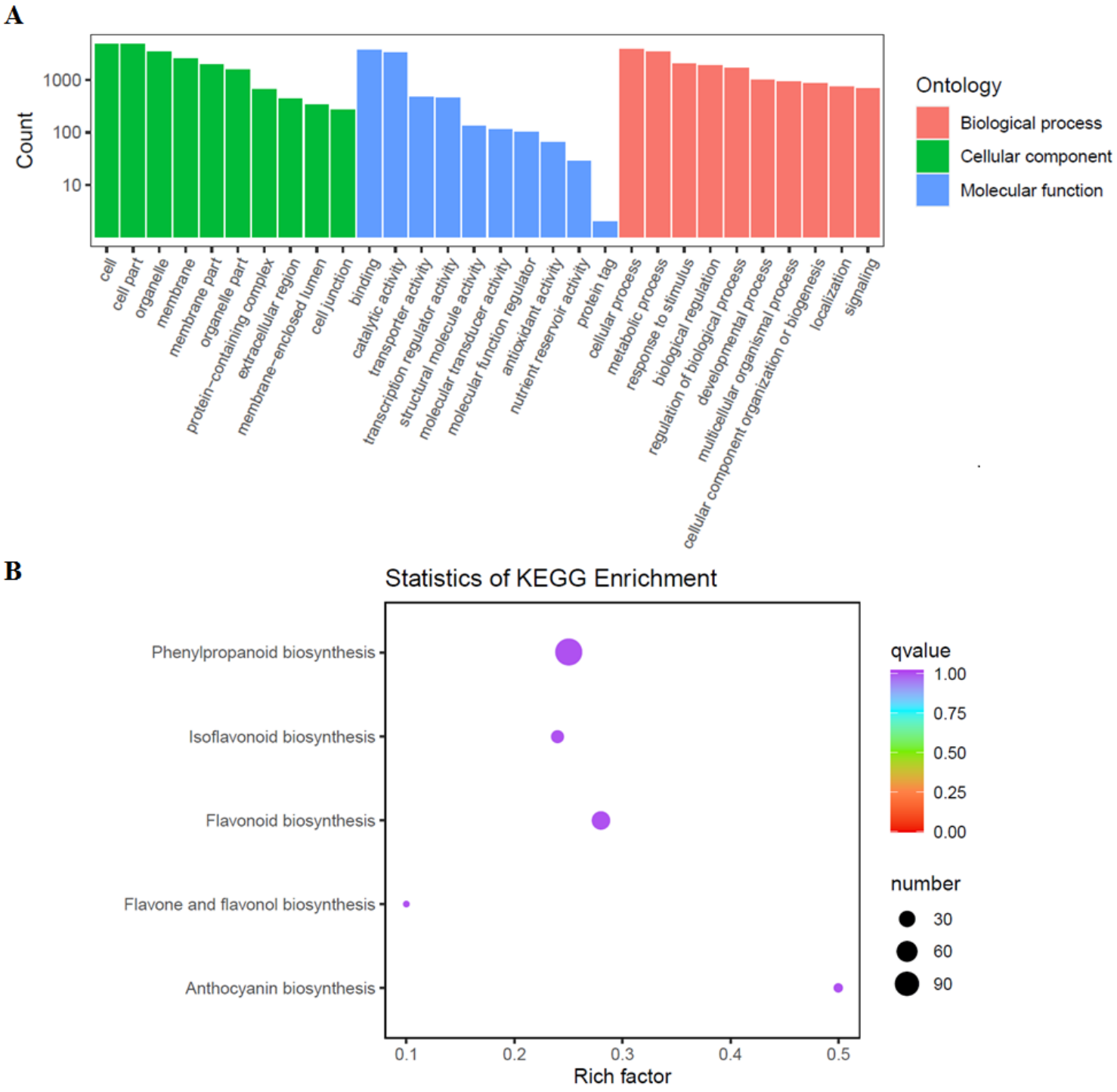

Figure 3

Transcriptome analysis of genes in SZS. (A) GO functional classification of genes. (B) KEGG pathway enrichment of the DEGs of flavonoid biosynthesis.

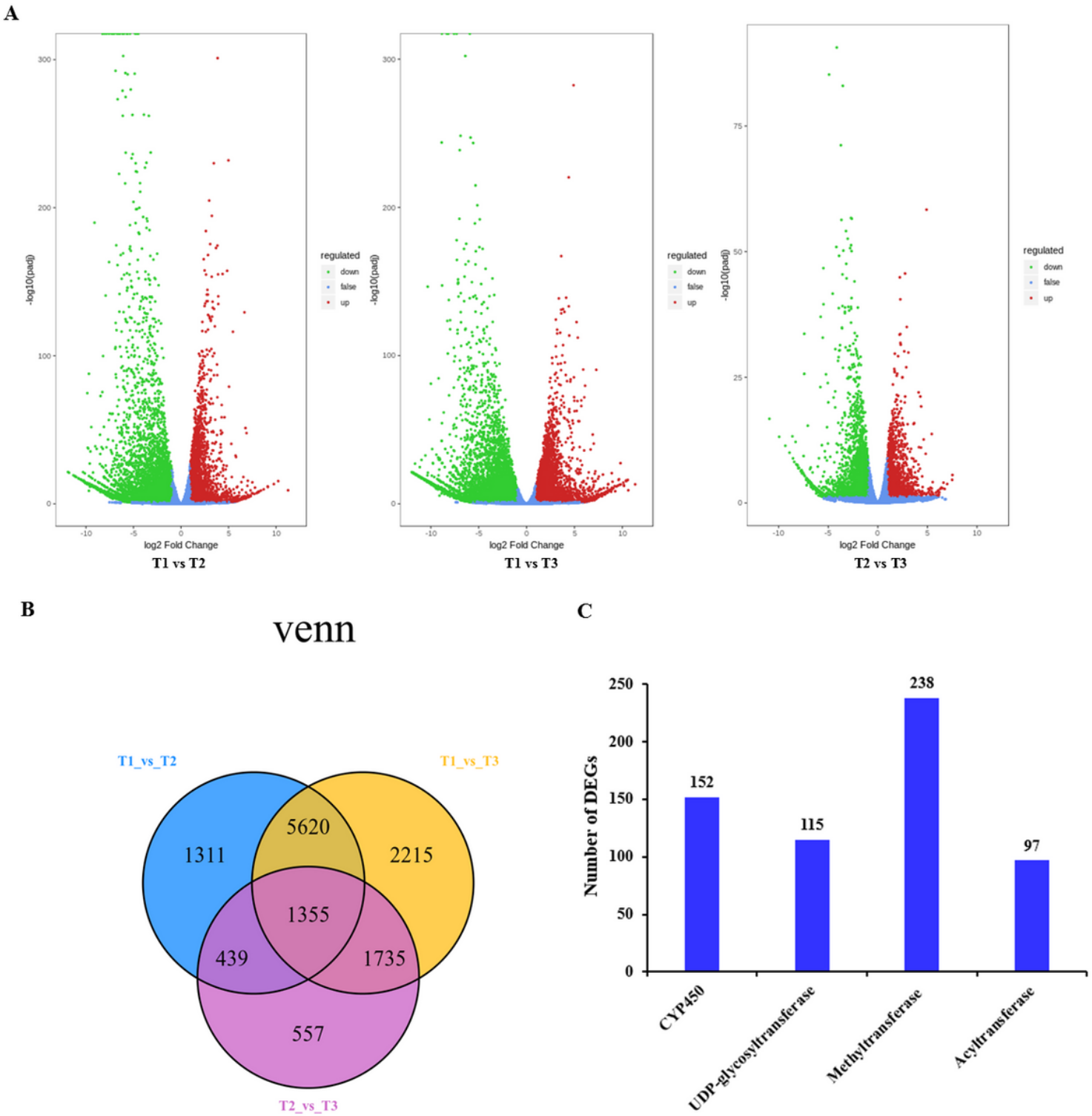

Figure 4

Analysis of differentially expressed genes in SZS. (A)Volcano plot of DEGs between T1, T2 and T3. (B) Venn diagram of DEGs at the three different growth stages of SZS. (C)The number of DEGs involving in flavonoid biosynthesis pathway.

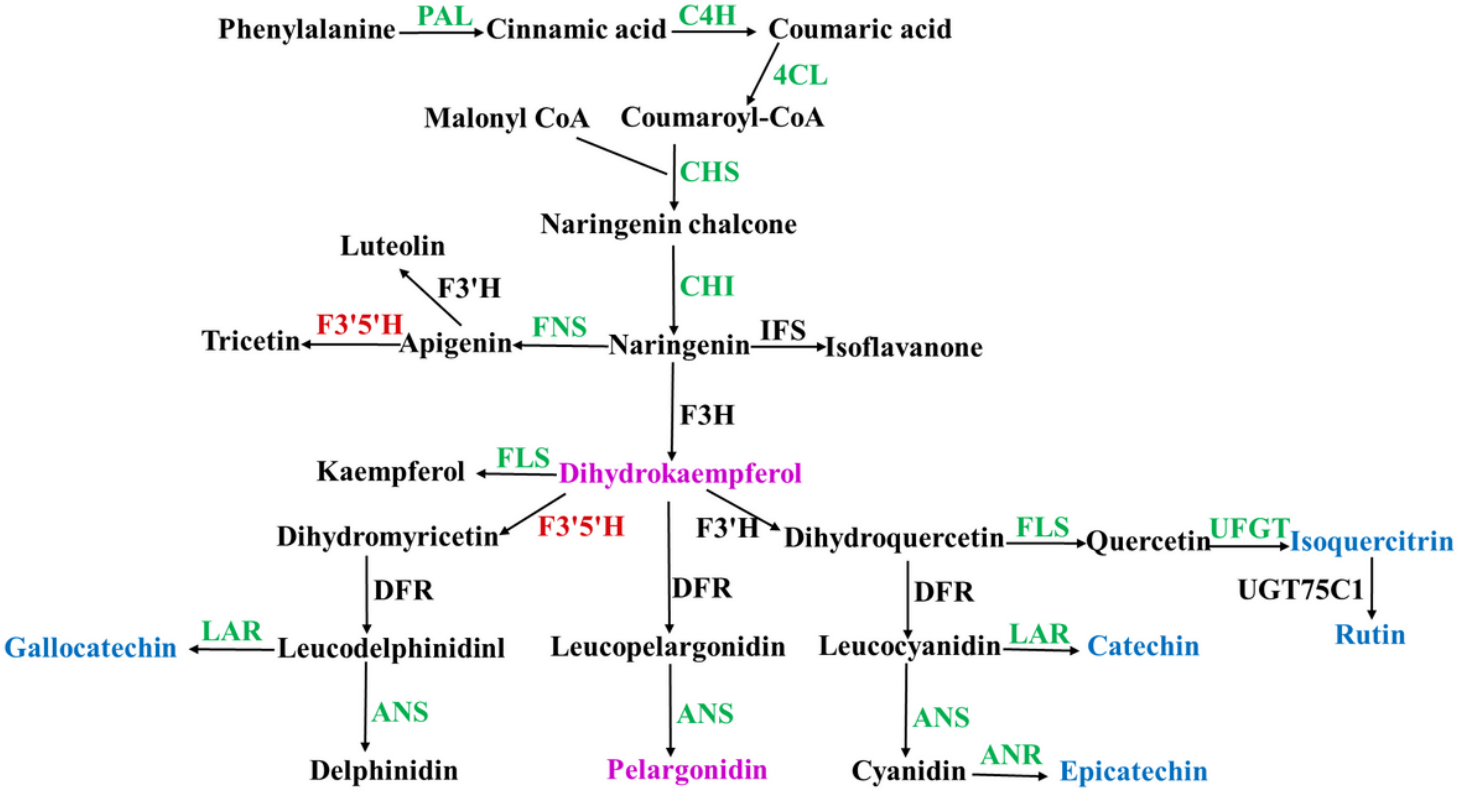

Figure 5

Biosynthetic pathway of flavonoids in SZS. Upregulated genes were in red, and downregulated genes were in green. Blue indicates a lower content of metabolites and purple indicates a higher content of metabolites in T3 than in T1.

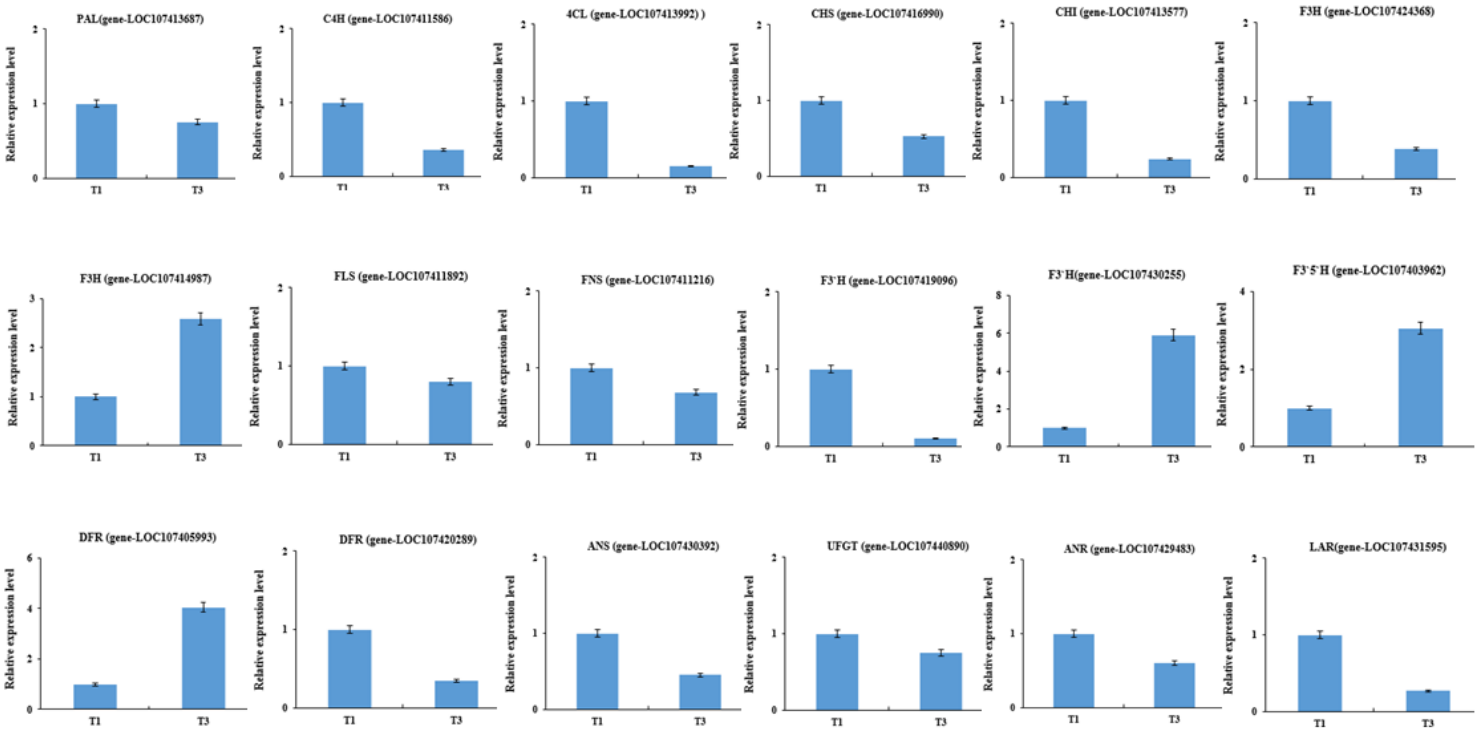

Figure 6

Relative expression levels of 18 genes in flavonoid biosynthetic pathway by qRT-PCR analysis. Data are presented as means ± standard deviations (n = 3).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1FlavonoidmetabolitesinSZS.xlsx
- TableS2GeneexpressionannotationinSZS.xlsx
- TableS3Specificprimersforgenesinvolvedinflavonoidsbiosynthesis.xlsx