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Transcriptome sequencing flower petals reveals insights into regulation of flavonoid biosynthesis in *Osmanthus fragrans*

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Abstract

Osmanthus fragrans Lour., one of the top 10 most popular flowers in China, is known for both its beauty and fragrance. It is rich in flavonoids, a class of secondary metabolites with significant neuroprotective, free-radical scavenging, and anti-oxidant activity. To understand the mechanisms regulating flavonoid biosynthesis, we conducted transcriptome sequencing *O. fragrans* flowers to analyze gene expressions during the full flowering stage. The RNA was isolated separately from petals of cvs. Yingui and Dangui, which were treated or not with jasmonic acid, salicylic acid, or abscisic acid. A total of 142 029 unigenes were *denovo* assembled, and 50 918 unigenes were annotated. The differentially expressed genes were identified, annotated, and classified. The results of transcriptome sequencing and real-time PCR revealed higher expressions of *phenylalanine ammonia-lyase* (*PAL*), *PAL1*, *chalcone synthase* (*CHS*), *flavanone-3-hydroxylase* (*F3H*), *flavonol synthase* (*FLS*), and lower expressions of *dihydroflavonol-4-reductase (DFR*), *anthocyanidin synthase* (*ANS*) in 'Yingui' than in 'Dangui'. Such an expression pattern facilitated the higher accumulation of flavonoids in 'Yingui'. Several genes of the flavonoid biosynthesis pathway were upregulated by jasmonic acid and salicylic acid in both the cultivars leading to flavonoid accumulation in their petals. In the *v-myb avian myeloblastosis viral oncogene homolog 1*(*MYB1*)-overexpressing petals, the expressions of *PAL*, *PAL1*, *CHI*, and *FLS* increased. The results suggest that MYB1 may participate in the flavonoid biosynthesis pathway and regulate the expression of some upstream genes in *O. fragrans*.

Additional key words:abscisic acid, DEGs, jasmonic acid, MYB transcription factor, salicylic acid.

Introduction

Flavonoids are secondary metabolites synthesized by the flavonoid biosynthetic pathway that bestow color to most flowers, fruits, and seeds of plants (Koes *et al*. 2005). They also play key roles in other plant processes including signaling between plants and microbes (Winkel-Shirley 2001), defense against cold or UV stresses, pathogen attacks, and plant diseases (Debeaujon *et al*. 2001, Peters and Constabel 2002).

The biosynthetic pathways of flavonoids have been well established, and the respective genes have been mostly isolated (Winkel-Shirley 2001, Tanaka *et al*. 2008, 2010). Suppression of the anthocyanidin synthase gene in *Torenia* × *hybrida* by RNAi yields white flowers (Nakamura *et al.* 2006), and suppression of chalcone isomerase gene in transgenic tobacco plants alters flavonoid components and color in both petals and pollen (Nishihara *et al.* 2005). Suppression of the flavanone-3-hydroxylase gene results in a complete loss of the original orange/reddish color and emission of methyl benzoate in carnations (Zuker *et al.* 2002). A higher expression of the *flavone synthase* (*FNS*) gene and/or suppression of the *dihydroflavonol-4-reductase* (*DFR*) gene may be necessary to achieve a higher flavone/anthocyanin ratio and obtain a bluer petunia by co-pigmentation (Tsuda *et al.* 2004). Expression of the gerbera *DFR* gene and suppressions of two endogenous genes *flavonol synthase* (*FLS*) and *flavonoid-3'-hydroxylase* (*F3'H*) increase the accumulation of pelargonidin pigments in tobacco flowers (Nakatsuka *et al.* 2007). Retransformation of a transgenic plant expressing gerbera *DFR* and suppression of flavonoid-3',5'-hydroxylase (F3'5'H) activity results in

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Abbreviations: ANS - anthocyanidin synthase; CHI - chalcone isomerase; CHS - chalcone synthase; DEGs - differentially expressed genes; DFR - dihydroflavonol 4-reductase; F3H - flavanone-3-hydroxylase; F3'H - flavonoid-3'-hydroxylase; FLS - flavonol synthase; MYB - v-myb avian myeloblastosis viral oncogene homolog; PAL - phenylalanine ammonia-lyase; PAL1- phenylalanine ammonia-lyase 1; R2R3 MYB - two repeats MYB domains.

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accumulation of predominantly pelargonidin derivatives in *Osteospermum hybrida* flowers (Seitz*et al.* 2007).
The regulation of the flavonoid pathway has been studied extensively in several plants (Broun 2005, Koes *et al.* 2005, Ramsay and Glover 2005). The two repeats of MYB domain (R2R3 MYB**)** transcription factors regulate gene expression by binding to the regulatory elements in the promoters of structural genes related to flavonoid biosynthesis (Koes *et al.* 2005). Expressions of two maize R2R3 MYB transcription factor genes *leaf color* (*LC*) and *colorless 1* (*C1*) in tomato are sufficient to upregulate the flavonoid pathway in fruit flesh (Bovy *et al.* 2002). Overexpression of a tomato MYB transcription factor gene *activation tagged insertion lines 1* (*ANT1*) upregulates genes encoding proteins in the anthocyanidin biosynthesis, which results in purple spotting on fruit epidermis and pericarp (Mathews *et al.* 2003). It has been proved that MYB transcription factors participate in the regulation of the flavonoid pathway in maize (Hernandez *et al*. 2004), snapdragon (Schwinn *et al.* 2006), petunia (Spelt *et al.* 2000, Quattrocchio *et al.* 2006), *Ipomoea* (Morita *et al.* 2006), tomato (Mathews *et al.* 2003), apple (Takos *et al.* 2006, Espley *et al.* 2007, Vimolmangkang *et al.* 2013), and *Arabidopsis* (Quattrocchio *et al.* 1999, Nesi *et al.* 2001, Baudry *et al.* 2010, Gonzalez *et al.* 2010, Mondal *et al.* 2017).
Osmanthus fragrans, also known as sweet osmanthus, is one of the top 10 domesticated flowers and a popular landscaping plant. It is valued for its beauty and fragrance and has been cultivated for over 2 500 years in China. Its numerous cultivars are classified into four cultivar groups Yingui, Jingui, Dangui, and Sijigui based on different flowering seasons, flower colors and inflorescence types (Xiang and Liu 2007, He *et al*. 2017). In the present study, we performed transcriptome sequencing for sweet osmanthus petals using the *Illumina* sequencing platform. The aim of this work was to determine if differences in the gene expressions of the flavonoid biosynthesis pathway is responsible for the different content of flavonoids in petals of 'Yingui' and 'Dangui'. The results could advance our understanding of flavonoid metabolism and contribute to utilization of flavonoid compounds and cultivation of new cultivars of sweet osmanthus.

Materials and methods

Plants and cultivation: Freshly cut flowering branches of *Osmanthus fragrans* Lour. (cv. 'Baijie' from the Yingui group and cv. 'Chenghong Dangui' from the Dangui group) were incubated under a 12-h photoperiod, an irradiance of 80 μmol $m^{-2}s^{-1}$, a constant temperature of 22 °C, and a relative humidity of 70% (Han *et al*. 2016, 2019). The flowering branches of both cultivars 'Chenghong Dangui' (D) and 'Baijie' (Y) were divided into four groups. One group of branches was not treated (D0 and Y0) and the three remaining groups were treated with 50 μM jasmonic acid (JA; DJ and YJ), 5 mM salicylic acid (SA; DS and YS), or 20 μM abscisic acid (ABA; DA and YA) for 3 h. The flowers at full flowering stage were collected and frozen in liquid nitrogen and stored at -80 °C until use.

Extraction of RNA: Total RNA was isolated from petals using a plant RNA kit (*Transgen Biotech*, Beijing, China). The RNA concentration was determined by a spectrophotometer (*Nanodrop Technologies*, Wilmington, DE, USA), and RNA integrity was evaluated by an *Agilent 2100* bioanalyzer (*Agilent Technologies*, Santa Clara, CA, USA) (more detail in Han *et al*. 2014b).

Library construction and transcriptome sequencing: A total amount of 3μg of RNA per sample was used. Sequencing libraries were generated using an *NEBNext® Ultra™* RNA library preparation kit for *Illumina®* (*NEB*, Ipswich, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. The mRNA was isolated from petals and purified using poly-T oligo-attached magnetic beads. The first cDNA strand was produced using random hexamer primers and reverse transcription kits. The second cDNA strand was synthesized by DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, *NEB* Next adapter oligonucleotides with a hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150-200 bp in length, the library fragments were purified with an *AMPure XP* system (*Beckman Coulter*, Beverly, USA). Then, 3 mm^3 of the uracil-specific excision reagent (USER) enzyme (*NEB*) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then, PCR was performed with phusion high-fidelity DNA polymerase, universal PCR primers, and an index primer. At last, PCR products were purified (the *AMPure XP* system) and library quality was assessed on an *Agilent Bioanalyzer 2100* system. Clustering index-coded samples was performed on a *cBot Cluster Generation* system using a *TruSeq PE Cluster*kit *v3-cBot-HS* (*Illumina*) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an *Illumina* platform and paired-end reads were generated.

Sequence data assembly and functional annotation: To obtain high quality data for sequence assembly and analysis, raw data (raw reads) in *fastq* format were firstly processed through in-house *perl* scripts. In this step, clean data (clean reads) were obtained by removing reads containing an adapter, reads containing ploy-N and low quality reads with Q < 20 from raw data using the *TRINITY* (Grabherr *et al*. 2011) RNASEQ_ROOT/util/normalize_by_kmer_coverage. The pl. transcriptome assembly was accomplished using *TRINITY* with a 'min_kmer_cov' set to 2 by default and all other parameters set default.

Unigene sequences were subjected to the *Blastx* algorithm of *Nr* (*NCBI* non-redundant protein sequences), *Nt* (*NCBI* non-redundant nucleotide sequences), *Swiss-Prot* (a manually annotated and reviewed protein sequence database) (Bairoch and Apweiler 1999), *COG/KOG*

(clusters of orthologous groups of proteins) (Sonnhammer and Koonin 2003, Tatusov*et al*. 2003), and *KEGG* ortholog database (Kanehisa *et al*. 2004) to obtain a protein with the highest similarity. Thus, we obtained the annotation information of protein function. The *KEGG* database was used to assign unigenes to pathways.

Unigene differential expression analysis: Differential expression analysis was performed using the *DESeq2R* package (Love *et al*. 2014) for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *P* values were adjusted using the Benjamini and Hochberg's approach for controling the false discovery rate. Genes with an adjusted *P*-value <0.01 found by *DESeq2* were assigned as differentially expressed. The expressions of unigenes were calculated using the fragments per kilobase million (FPKM) method. The expressions of differentially expressed genes (DEGs) were computed by the following formula: FPKM = cDNA fragments/[mapped fragments (millions) × transcript length (kb)]. The FPKM results can be directly used to compare gene expression within differential samples.

Isolation and sequence analysis of *OfMYB1*: For isolation of *OfMYB1* from *O. fragrans*, two primers MYB1f and MYB1r (Table 1 Suppl.) were synthesized to amplify the full length *MYB1* gene. A PCR amplification was performed with the primers. After an initial 94 °C 4 min denaturation step, 35 cycles were run, each with 45 s of denaturation at 94 °C, followed by 45 s annealing at 56 °C, and 60 s extension at 72 °C. The PCR product was cloned into the pMD19-T vector (*TaKaRa*, Dalian China) and sequenced. We then compared the *amino acid sequence* of OfMYB1 with MYBs from other plant species. These sequences were aligned using *Clustal W v. 1.83*. Phylogenetic analysis was performed using *MEGA 4.1.* A neighbor joining tree was constructed according to the distance matrix that had been computed.

Real-time quantitative PCR analysis was utilized to evaluate the relative expressions of related genes. Gene specific primers were designed using the *Primer 5.0* software (*Primer Biosoft International*, Palo Alto, CA, USA) and listed in Table 1 Supp l. Each 20 mm^3 of reaction included 10 mm^3 of *SYBR Green qRT-PCR* mix. The following amplification program was used: the initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 7 s, annealing at 60 °C for 10 s, and extension at 72 °C for 50 s. The sweet osmanthus β-actin gene was used as an internal reference, and zelative expressions were computed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Each sample was prepared in three to five biological replicates. The *SPSS v. 12.0* (*SPSS Inc.*, Chicago, IL, USA) software was used for statistical analysis.

Transient overexpression of *OfMYB1* in *O. fragrans* flower petals: Transient overexpression of *OfMYB1* (accession No. KM504383) in petals of *O. fragrans* 'Dangui' in the full flowering stage was performed according to Han *et al*. (2016). The *MYB1* coding region was amplified using gene specific primers (MYB1f1 and MYB1r1) (Table 1 Suppl.) and subcloned into the pHBT vector. The resulting HBT-*OfMYB1* vector (35Spro:MYB1-GFP) and HBT vector (35Spro:GFP, control) were transformed into *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw method. After overnight culture, the *Agrobacterium* was resuspended in 10 mM $MgCl_2$, 10 mM MES, 150 mM acetosyringone until reaching an absorbance of 0.6 at 600 nm. The petals were cut into a 2 mm size, vacuum infiltrated with *A. tumefaciens* for 15 min, and kept in the dark in 5% (m/v) sucrose for 36—48 h. The RNA extracted from infiltrated petals was analyzed using real-time quantitative PCR (qPCR) (Han *et al*. 2016).

Results

After filtering the raw sequences, 27 475 198 (in D0), 24 547 401 (in DJ), 26 079 942 (in DS), 27 722 398 (in DA), 28 420 554 (in Y0), 31 683 095 (in YJ), 34 016 142 (in YS), and 27 722 398 (in YA) high quality reads were obtained (Table 2 Suppl.). Using the *TRINITY* assembly software, 142 029 unigenes were assembled with an N_{50} length of 1 300 bp. The length distributions of contigs, transcripts, and unigenesare shown in Table 3 Suppl. Transcriptome sequencing data have been deposited in the *NCBI* sequence read archive (*SRA*) database with accession number PRJNA565564.

The highest number of upregulated (2 223) and downregulated (2 171) unigenes was observed between Y0 and D0. The lowest number of upregulated (896) unigenes was observed between D0 and DJ, and the lowest number of downregulated (638) unigenes was observed between Y0 and YA (Table 4 Suppl.). There were 494 overlap DEGs between Y0 and YJ, Y0 and YS, Y0 and YA and Y0 and D0 combinations, and 684 overlap DEGs between D0 and DJ, D0 and DS, D0 and DA and Y0a and D0 combinations (Fig. 1 Suppl.). The results suggest that the expression of 494 and 684 genes were influenced by the three plant hormones (JA, SA, and ABA) in 'Yingui' and 'Dangui', respectively.

A total of 50 918 unigenes were annotated by sequence-based alignments in *COG*/*KOG* (Sonnhammer and Koonin 2003, Tatusov *et al*. 2003), *GO* (Harris *et al.* 2004), *KEGG* (Kanehisa *et al*. 2004), *Pfam* (Finn *et al*. 2007), *Swiss-Prot* (Bairoch and Apweiler 1999), *NCBI*, and *NR* databases (https://www.ncbi.nlm.nih.gov/) (Table 5 Suppl.) using the *BLASTX* algorithm (Altschul *et al*. 1990) with an E-value less than $1e^{-5}$ demonstrating a high sequence homology.

Unigene sequences were subjected to the *BLASTX* algorithm of *Nr*, *Nt*, *Swiss-Prot*, *COG/KOG*, and the *KEGG* ortholog database to obtain a protein with the highest similarity; we obtained the annotation information of protein function. The *KEGG* database was used to assign unigenes to pathways.

Of the 50 918 unigenes, 26 941 unigenes were annotated in the GO database, and their functions were

Table 1. The number of annotated unigenes.

DEG Set	Annotated	*COG*	*GO*	*KEGG*	*KOG*	*Pfam*	*Swiss-Prot*	*Nr*
D0_*vs*_DJ	1448	463	822	436	682	1126	1096	1439
D0_vs_DS	1962	610	1096	571	968	1502	1488	1951
D0_*vs*_DA	2184	651	1205	633	1000	1681	1647	2173
Y0_*vs*_D0	3305	1027	1851	1100	1790	2394	2336	3272
Y0_*vs*_YJ	1689	563	1013	537	852	1309	1287	1681
Y0_*vs*_YS	1727	565	984	558	893	1342	1308	1722
Y0_*vs*_YA	1538	490	873	484	725	1188	1154	1528

Table 2. Identified flavonoid synthesis pathway related unigenes.

Unigene ID	'Dangui' *vs.*'Yingui' log2FC	regulated	Nucleotide length	Protein length	Nr_annotation	Accession number
BMK.70848	0.20407	up	2136 bp	712 aa	phenylalanine ammonia-lyase [*Olea europaea*]	MN512442
CL35673	2.10227	up	2139 bp	713 aa	phenylalanine ammonia-lyase 1[*Nelumbo nucifera*]	MN512443
CL859	5.17495	up	1170 bp	390 aa	chalcone synthase [*Olea europaea*]	KR604813
CL35874	0.58434	up	747 bp	249 aa	chalcone isomerase [*Olea europaea*]	ALL27265
CL36577	4.23624	up	1101 bp	367 aa	flavanone 3-hydroxylase [*Gossypium hirsutum*]	MN512444
CL40977	5.71978	up	1002 bp	334 aa	Flavonol synthase [*Petunia x hybrida*]	MN512445
BMK.70048	-1.704667	down	1128 bp	376 aa	dihydroflavonol reductase [*Forsythia x intermedia*]	ANA96271
BMK.46512	-2.670299	down	1146 bp	382 aa	anthocyanidin synthase [*Forsythia* × *intermedia*]	MN512446
CL35948	0.15411	up	585 bp	195 aa	Myb-related protein 305 (*Antirrhinum majus*)	KM504383

divided into biological process, cellular component, and molecular function (Fig. 2 Suppl.). The largest number of annotated genes in the three functions were detected in the Y0-*vs*-D0 combination: 'biological_process' (GO:0008150) comprised 20 848 unigenes (40.94%), 'cellular_component' (GO:0005575) included 13 325 unigenes (26.17%), and 'molecular_function' (GO:0003674) consisted of 22 357 unigenes (43.91%). Among the seven DEG sets, the Y0-*vs*-D0 combination had the largest number of annotated genes (3 305), and 1 027 DEGs were aligned to the *COG* database (Table 1).

The unigenes annotated to the *COG* database were classified into 25 different functional classes in Y0-*vs*-D0 (Fig. 3 Suppl.). The cluster of general function prediction (276; 18.02%) presented the largest group followed by replication, recombination, and repair (160; 10.44%), transcription (134; 11.9%), signal transduction mechanisms (125; 12.28%), carbohydrate transport and metabolism (116; 7.57%), posttranslational modification, protein turnover, chaperones (102; 6.66%), and amino acid transport and metabolism (102; 6.66%).

A total of 16 985 unigenes were annotated to the *KEGG* database, and 1 091 DEGs were assigned to 117 different *KEGG* pathways. The *KEGG* categories were mainly classified into five groups: cellular process, environmental information processing, genetic information processing, metabolism, and organismal systems. The greatest number of DEGs were in starch and sucrose metabolisms (ko03010, 44 DEGs, 3.14%) followed by biosynthesis of amino acids (39), plant hormone signal transduction (38), protein processing in endoplasmic reticulum (37), carbon metabolism (33), and phenylpropanoid biosynthesis (26) (Fig. 4 Suppl.).

Flavonoid accumulation can be regulated by the expressions of flavonoid biosynthesis genes. Using the analysis platform (http://www.biomarker.com.cn/biocloud), eight DEGs were selected for further analysis. These DEGs were the key genes related to flavonoid biosynthesis. Of those, six DEGs were upregulated: BMK.70848 (PAL), CL35673 (PAL1), CL859 (CHS), CL35874 (CHI), CL36577 (F3H), and CL40977 (FLS), and two DEGs were downregulated: BMK.70048 (DFR), and BMK.46512 (ANS) in 'Yingui' (Table 2, Figs. 1, 2). The results of the gene expression profiles of other DEG sets exhibited small differences in transcriptions (Fig. 1).

Sequence analysis of OfMYB1 shows that a unigene

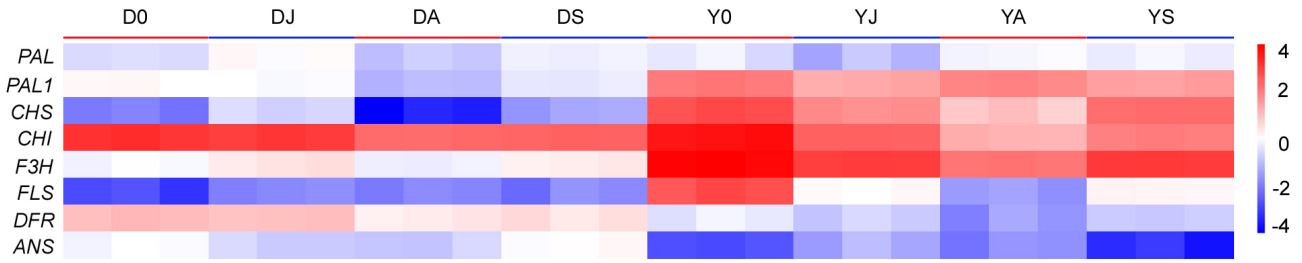

Fig. 1. A heat-map depicting normalized log2-fold changes in mRNA expression inferred from RNAseq data for transcripts involved in flavonoid biosynthesis. DO, DJ, DA, DS, YO, YJ, YA, and YS, please refer to Materials and methods section. PAL - phenylalanine ammonia-lyase, PAL1 - phenylalanine ammonia-lyase 1, CHS -chalcone synthase, CH -chalcone isomerase, F3H -flavanone-3-hydroxylase, FLS -flavonol synthase, DFR - dihydroflavonol-4-reductase, ANS -anthocyanidin synthase.

CL35948 was slightly upregulated in 'Yingui'. Sequence comparison indicates a high similarity between this unigene and the one encoding *Antirrhinum majus* myb-related protein 305. The unigene appeared to be a full length sequence of 585 bp encoding a polypeptide of 195 amino acid residues and named *O. fragrans* MYB1 (OfMYB1). Sequence alignment reveals that MYB1 shared a high amino acid sequence identity with *Antirrhinum majus* myb305 (AmMYB305), *Coffea eugenioides* MYB305, *Sesamum indicum* MYB305, *Gerbera* hybrid cultivar MYB305, *Petunia* × *hybrida* EOBI, *Mucuna pruriens* MYB305, *Vitis vinifera* MYB24, and *Capsicum baccatum* MYB21 (Fig. 5 Suppl.). A phylogenetic tree was constructed based on the amino acids of OfMYB1 and some MYBs from other plant species. The OfMYB1 shows a high similarity with *Antirrhinum majus* MYB305, *Coffea eugenioides* MYB305, and *Sesamum indicum* MYB305 (Fig. 5 Suppl.).

To investigate whether the results of gene expression profiles obtained from RNA-seq were accurate, the relative expressions of the eight genes related to the flavonoid biosynthesis pathway were examined by real-time qPCR (Fig. 3). Compared with 'Dangui', the transcriptions of *PAL1*, *CHS*, *F3H*, and *FLS* in petals of 'Yingui' increased

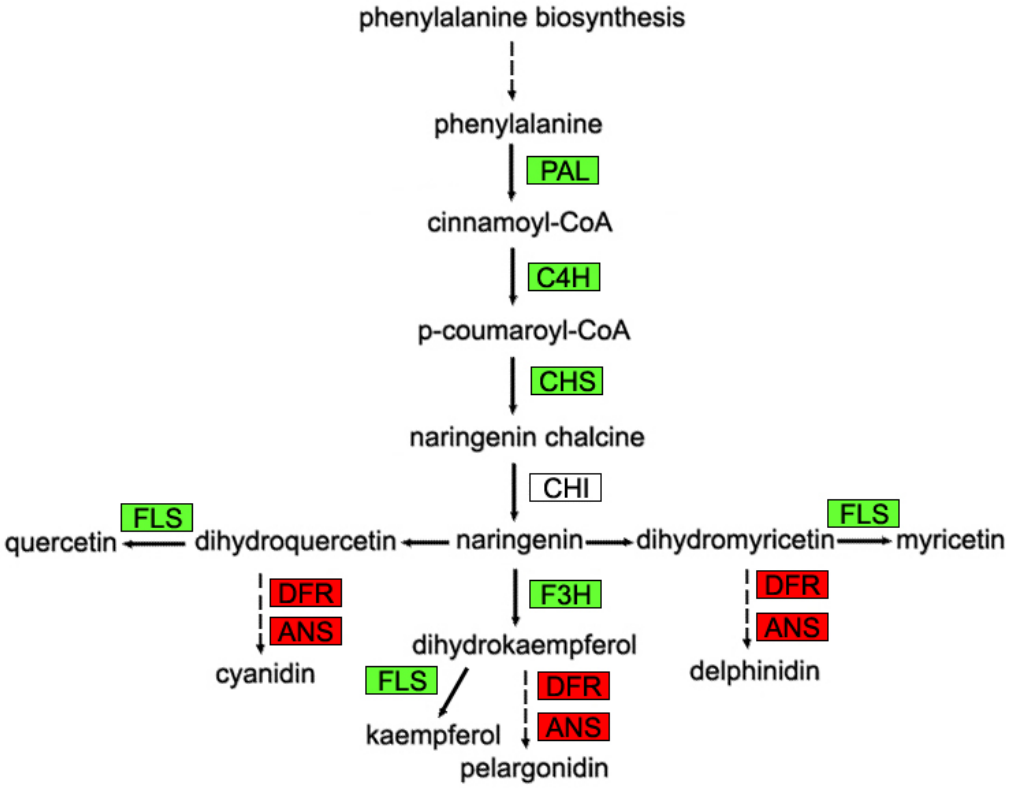

Fig. 2. Differentially expressed genes in flavonoid biosynthesis pathway (Y0 and D0). Y0 -the full flowering stage petals of 'Yingui'; D0 -the full flowering stage petals of 'Dangui'. *Red* represents upregulated genes and *green* represents downregulated genes. PAL - phenylalanine ammonia-lyase, C4H -cinnamate-4-hydroxylase, CHS -chalcone synthase, CHI -chalcone isomerase, F3H -flavanone-3-hydroxylase, FLS -flavonol synthase, DFR -dihydroflavonol-4-reductase, ANS -anthocyanidin synthase.

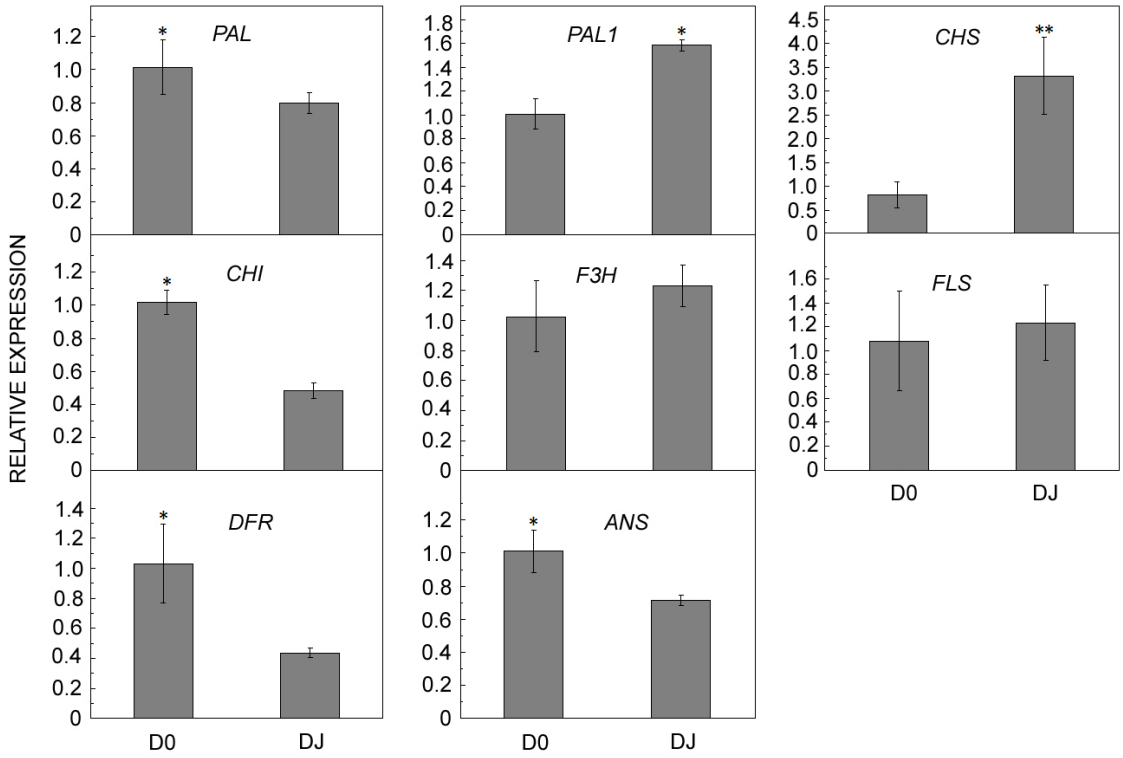

Fig. 3. Transcriptions of genes involved in flavonoid biosynthesis pathway in Y0 and D0. Y0 -the full flowering stage petals of 'Yingui', D0 -the full flowering stage petals of 'Dangui'. For abbreviations see Fig. 2. Means ± SDs, $n = 3$. Statistically significant differences at * - $P<0.05$ and ** - $P<0.01$.

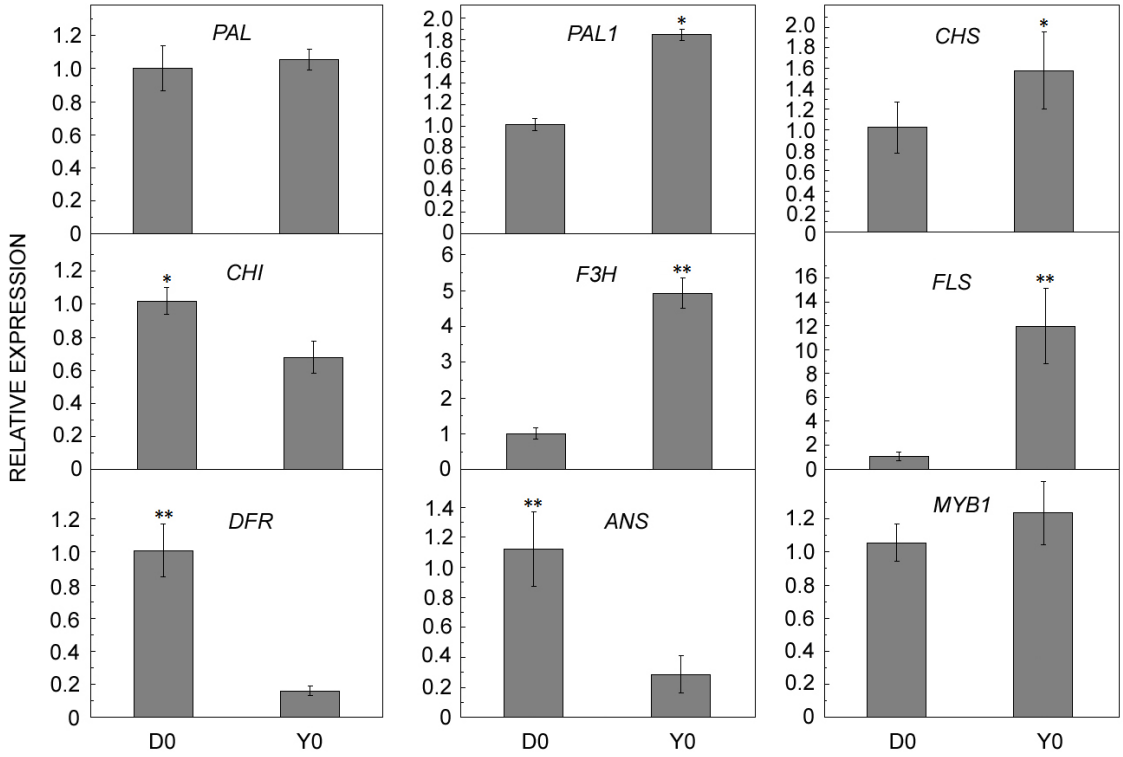

Fig. 4. Transcriptions of genes involved in flavonoid biosynthesis pathway in D0 and DJ. D0 -the full flowering stage petals of 'Dangui', DJ -the full flowering stage petals of 'Dangui' treated with JA. For abbreviations see Fig. 2. Means ± SDs, $n = 3$. Statistically significant differences at * - $P<0.05$ and ** - $P<0.01$.

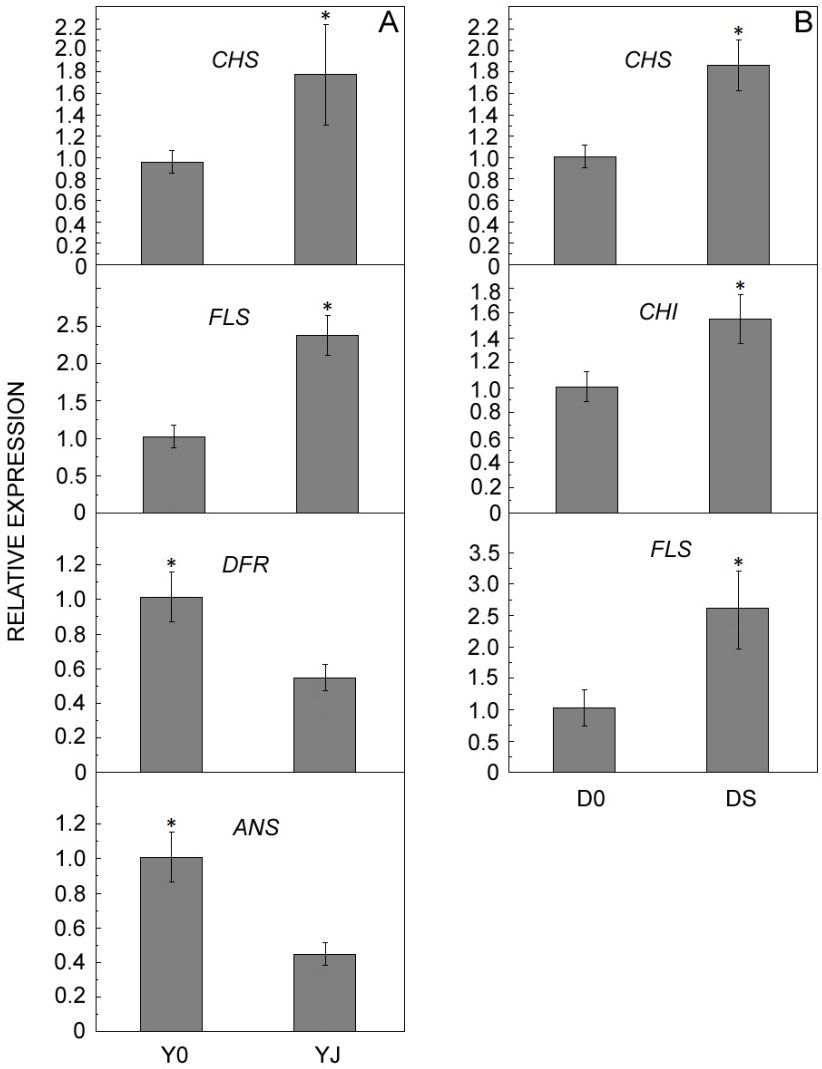

Fig. 5. Transcriptions of some genes involvedin flavonoid biosynthesis pathwayin Y0 and YJ (*A*) and D0 and DS (*B*). Y0 -the full flowering stage petals of 'Yingui' , YJ -the full flowering stage petals of 'Yingui' treated with JA; D0 -the full flowering stage petals of 'Dangui',DS -the full flowering stage petals of 'Dangui' treated with SA. For abbreviations see Fig. 2. Means ± SDs, $n = 3$. Statistically significant differences at * - $P<0.05$.

0.8-, 0.5-, 3.9-, and 10.9-fold, respectively, and those of *CHI*, *DFR*, and *ANS* were reduced 0.4-, 5.3-, and 2.9-fold, respectively. Moreover, there was no significant difference in the expression of *PAL* between 'Yingui' and 'Dangui' (Fig. 3). Thus, the qPCR analysis corroborated the expression patterns of the eight genes inferred by RNA-seq. Both RNA-seq and qPCR analysis show that the expression of *OfMYB1* in 'Yingui' petals was slightly higher than in 'Dangui' (Table 2, Fig. 3).

To determine the effects of hormones on the expressions of these genes, the flowers treated with JA, SA, and ABA for 3h were examined by real-time qPCR. According to the RNA-seq results, *PAL1*, *CHS*, *F3H*, and *FLS* were upregulated, and *PAL1*, *CHI*, *DFR*, and *ANS* were slightly downregulated after the treatment with JA (Fig. 1). In DJ, the transcriptions of *PAL1*, *CHS*, *F3H*, and *FLS* increased 0.58-, 2.43-, 0.21-, and 0.13-fold, and those of *PAL*, *CHI*, *DFR*, and *ANS* were reduced 0.2-, 0.52-, 0.58-, and 0.3-fold, respectively, when compared with those in D0 and as indicated by qPCR (Fig. 4). The transcriptions of the genes obtained by RNA-seq were consistent with those detected by the qPCR. As indicated by the qPCR results, *CHS* and *FLS* increased 0.81- and 1.35-fold, and *DFR* and *ANS* were reduced 0.45- and 0.55-fold in YJ, respectively,

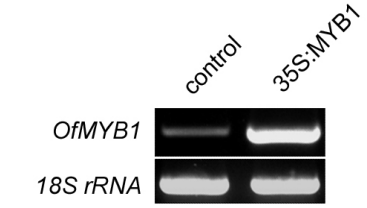

Fig. 6. Detection of *v-myb avian myeloblastosis viral oncogene homolog 1* (*MYB1*) transcripts in control and transiently transformed petals using reverse transcription polymerase chain reaction detection.

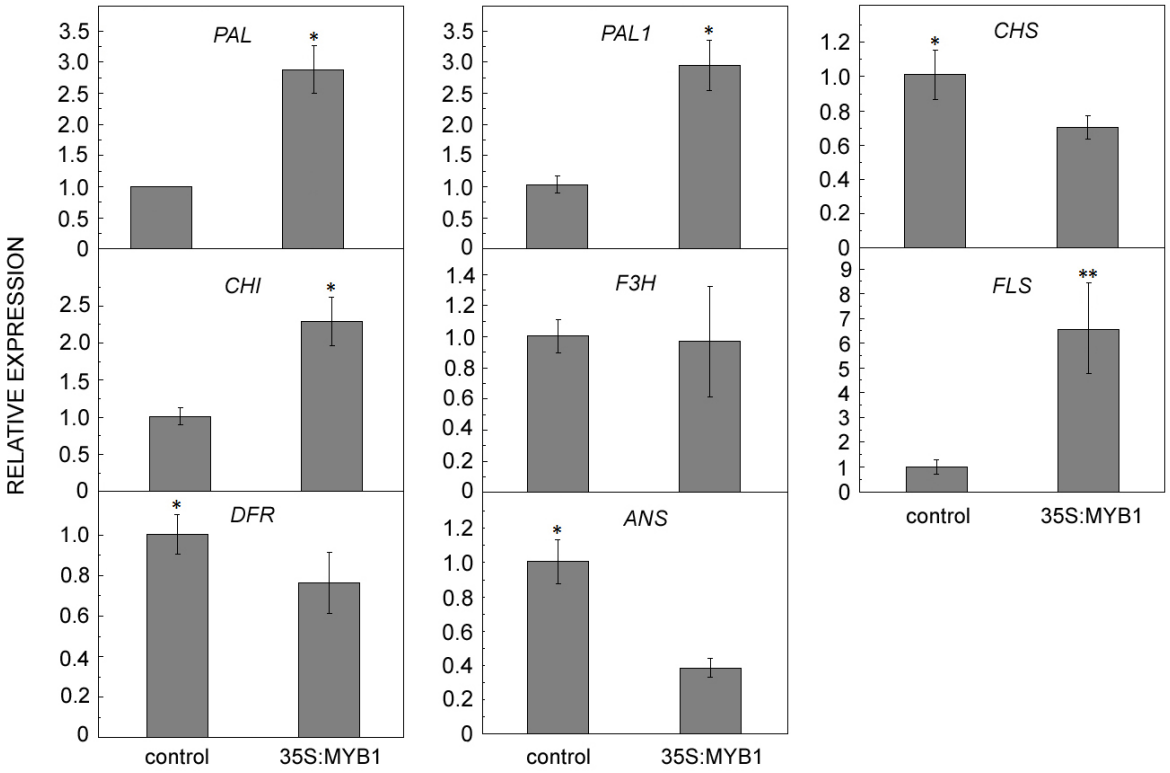

Fig. 7. Comparison of flavonoid biosynthesis pathway gene transcriptions in wild type 'Dangui' flower petals (control) and transiently transformed petals (35S:MYB1). For abbreviations see Fig. 2. Means ± SDs, $n = 3$. Statistically significant differences at * - $P < 0.05$ and ** - $P < 0.01$.

when compared with their expressions in Y0 (Fig. 5*A*). The transcriptions of *PAL*, *PAL1*, and *CHI* were not significantly different between Y0 and YJ. These results suggest that the upstream genes of the flavonoid biosynthesis pathway were upregulated and the downstream genes were downregulated promoting the accumulation of flavonoids in sweet osmanthus flowers treated with JA.

In flowers treated with SA, the transcriptions of *CHS*, *CHI*, and *FLS* increased 0.85-, 0.56-, and 1.58-fold, respectively, as indicated by qPCR (Fig. 5*B*). The transcriptions of the remaining genes were not significantly different between D0 and DS. These results suggest that the treatment of flowers with SA upregulated the transcription of some upstream genes of the flavonoid biosynthesis pathway, resulting in an increased flavonoid content. There were no significant changes in the expressions of the genes between the flowers before and after the treatment with ABA implying that ABA did not alter the flavonoid metabolism in flowers.

To determine the ability of *OfMYB1* to upregulate the transcriptions of the flavonoid biosynthesis-related genes, *OfMYB1* was transiently transformed into 'Dangui' flower petals. The analyses of flowers transformed with *35S:OfMYB1* and *35S:GFP* revealed an overexpression of *OfMYB1* in the former (Fig. 6). The transcriptions of *PAL*, *PAL1*, *CHI*, and *FLS* were affected by the *OfMYB1* overexpression: their expressions increased 1.8-, 1.9-, 1.3-, and 5.5-fold, respectively in *OfMYB1*-overexpressing petals compared with the control (Fig. 7).

Discussion

Transcriptome sequencing represents an attractive alternative to whole-genome sequencing because it analyzes only transcribed portions of the genomeand avoiding non-coding and repetitive sequences (Margulies *et al*. 2005, Huse *et al*. 2007, Novaes *et al*. 2008). The transcriptome provides information on gene expression, gene regulation, and amino acid content of proteins. Therefore, transcriptome analysis is essential to interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues (Wang *et al*. 2009, Wei *et al*. 2011). In this study, using the *TRINITY de novo* assembly program, short-read sequences were assembled into 128 098 unigenes with an N50 length of 882 bp and with a mean length of 599 bp. It was annotated 70 915 unigenes. The DEGs were identified, annotated, and classified. The datas will be a useful information for future molecular and genetic studies on sweet osmanthus. *Osmanthus fragrans*, a woody aromatic plant mainly distributed in China and famous for its beauty and fragrance, is rich in flavonoids. The accumulation of flavonoids is promoted by upregulation of *CHS*, *CHI*, *F3H*, and *FLS* genes. In the present study, we compared the expression patterns of these genes in 'Dangui' and 'Yingui' petals. The *PAL1*, *CHS*, *F3H*, and *FLS* expressions were higher and *DFR* and *ANS* expressions were lower in 'Yingui' than in 'Dangui' petals. The expression profiles reflected the increased synthesis of flavonoids. The *F3H* and

FLS, which increased 3.9- and 10.9-fold, respectively, in 'Yingui' flowers, are likely the two key genes in flavonoid biosynthesis regulation. The expressions of *DFR* and *ANS* were very low compared with those of *CHS*, *F3H*, and *FLS*, which explains the low capability of sweet osmanthus to synthesize anthocyanins. These results corroborate earlier studies on *O. fragrans*, which showed that the content of carotenoids is positively correlated with petal coloration (Han *et al*. 2013, 2014a). Thus, the anthocyanins are not the main pigments to determine flower color in sweet osmanthus.

Genetic and physiological evidence has shown that JA regulates the WD-repeat/bHLH/MYB complex-mediated anthocyanin accumulation in *Arabidopsis thaliana* (Qi *et al*. 2011). JA-inducible MYB14 leads to the accumulation of flavonoids in conifer trees (Bedon *et al*. 2010). A previous study showed that JA can induce the expression of some transcription factors (such as MYBs), thus promoting the expressions of genes related to the flavonoid biosynthesis pathway and accumulation of flavonoids. The genes *PAL*, *CHS*, *F3H*, and *FLS* were upregulated, and *DFR* and *ANS* were downregulated when the flowers were treated with JA, promoting the accumulation of flavonoids in sweet osmanthus flowers. These results are thus consistent with previous studies.

Salicylic acid can be used as a chemical elicitor to promote the synthesis of total flavonoids in a suspension culture of *Andrographis paniculata* (Mendhulkar *et al*. 2013). The expression of the *LcCHI* gene and the content of total flavonoids in the *Lycium chinense* berries are upregulated by SA treatment (Guan *et al*. 2014). In the present study, several upstream genes of the flavonoid biosynthesis pathway were upregulated whereas the expressions of downstream genes remained nearly constant when the flowers were treated with SA, promoting the accumulation of flavonoids in sweet osmanthus flowers. Salicylic acid may activate some transcription factors that regulate the flavonoid biosynthesis pathway.

The exogenous ABA enhances PAL activity and promotes the accumulation of flavonoids in *Ginkgo biloba* (Hao *et al*. 2010). Abscisic acid also controls ripening process and regulates the blue berry flavonoid biosynthesis pathway (Zifkin *et al*. 2012). In the present paper, ABA treatment had no significant effect on the expressions of the genes involved in the flavonoid biosynthesis pathway. These results are thus not consistent with other studies.

Flavonoid and anthocyanin biosynthesis pathways are regulated by MYB transcription factors. The MYBs regulating the flavonol branch of the flavonoid biosynthesis pathway have also been identified in *Arabidopsis* and grapevine (Mehrtens *et al*. 2005, Stracke *et al*. 2007, Czemmel *et al*. 2009). Our previous research showed that the expression profile of *OfMYB1* is not consistent with those of *PAL*, *CHS*, and *CHI* suggesting that MYB1 does not regulate these genes and flavonoid biosynthesis pathway (Han *et al*. 2015). However, the results of the yeast one-hybrid system showed that MYB1 can bind to the promoter of *OfPAL* and thus regulate its expression (Han *et al*. 2015). In the present paper, the results demonstrate that the overexpression of *MYB1* enhanced the expression of *PAL*, *PAL1*, *CHI*, and *FLS*, ultimately increasing the accumulation of flavonoids. The increased expression of *PAL* and *PAL1* promoted the biosynthesis of phenylpropanoids including some aromatic compounds, alkaloids, flavonoids, *etc*. However, the overexpression of *MYB1* did not affect other genes related to flavonoid biosynthesis. Taken together, these results suggest that MYB1 and some other transcription factors (*e.g*., MYBs and bHLH) may co-regulate the expression of phenylpropanoid metabolic pathway-related genes. Future inquiries should aim to discover additional transcription factors in order to elucidate the mechanism of flavonoid biosynthesis.

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