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

Y.J. HAN*, M.F. DONG, H.Y. WANG, X.D. WANG, K. LI, and F.D. SHANG*

School of Life Sciences, State Key Laboratory of Cotton Biology, State Key Laboratory of Crop Stress Adaptation and Improvement, Key Laboratory of Plant Stress Biology, Laboratory of Plant Germplasm and Genetic Engineering, Henan University, Kaifeng 475004, P.R. China

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 antioxidant activity. To understand the mechanisms regulating flavonoid biosynthesis, we conducted transcriptome sequencing of *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* (*F3'H*), *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 I* (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 (Nakatsu et al. 2007). Retransformation of a transgenic plant expressing gerbera *DFR* and suppression of flavonoid-3′,5′-hydroxylase (*F3′5′H*) activity results in
accumulation of predominantly pelargonidin derivatives in *Osteospermum hybridum* 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 (ANTI) 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 (Spyck 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, Vinolmangkang 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 2500 years in China. Its numerous cultivars are classified into four cultivar groups 'Yingui', 'Dangui', and 'Siijuji' 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 are 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 the 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 3 µg 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 denaturation 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 mm³ 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 the library quality was assessed on an Agilent Bioanalyzer 2100 system. Clustering indexed-codes 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 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*
Differential expression analysis was performed using the DESeq2 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 controlling 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 QMYB1: For isolation of QMYB1 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 QMYB1 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 Suppl. Each 20 mm³ of reaction included 10 mm³ 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 relative expressions were computed by the 2^-ΔΔCt 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.

Transparent overexpression of QMYB1 in O. fragrans flower petals: Transparent overexpression of QMYB1 (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-QMYB1 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 MgCl₂, 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% (v/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 DA), 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 N₅₀ length of 1 300 bp. The length distributions of contigs, transcripts, and unigenes are 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 was 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 (Sonhammer 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
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 (33,305), and 1027 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 (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).
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, *Coffea eugenioides* MYB305, *Sesamum indicum* MYB305, *Gerbera* hybrid cultivar MYB305, *Petunia × hybrida* EOB1, *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 OMYB1 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. 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.

**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, CH -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 ** - \( P<0.01 \).
FLAVONOID BIOSYNTHESIS IN FLOWERS of OSMANTHUS FRAGRANS

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. 5. Transcriptions of some genes involved in flavonoid biosynthesis pathway in Y0 and Y1 (A) and D0 and DS (B). Y0 - the full flowering stage petals of ‘Yingui’, Y1 - 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.

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.
when compared with their expressions in Y0 (Fig. 5A). 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. 5B). 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 \( OMYB1 \) to upregulate the transcriptions of the flavonoid biosynthesis-related genes, \( OMYB1 \) was transiently transformed into ‘Dangui’ flower petals. The analyses of flowers transformed with \( 35S:OMYB1 \) and \( 35S:GFP \) revealed an overexpression of \( OMYB1 \) in the former (Fig. 6). The transcriptions of \( PAL, PAL1, CHI, \) and \( FLS \) were affected by the \( OMYB1 \) overexpression: their expressions increased 1.8-, 1.9-, 1.3-, and 5.5-fold, respectively in \( OMYB1 \)-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 genome and avoids 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 data 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
FLAVONOID BIOSYNTHESIS IN FLOWERS OF OSMANTHUS FRAGRANS

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 expression 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 LeCHI gene and the content of total flavonoids in the Lycium chinense berries were 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 (Zilkin 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 OjMYB1 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 OjPAL 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.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J.: Basic local alignment search tool. - J. mol. Biol. 215: 403-410, 1990.

Bairouch, A., Apeweiler, R.: The SWISS-PROT protein sequence data bank and its supplement TrEMBL in 1999. - Nucl. Acids Res. 28: 49-54, 1999.

Baudry, A., Heim, M.A., Dubreucq, B., Caboche, M., Weisshaar, B., Lepiniec, L.: TT2, TT8, and TTG1 synergistically specify the expression of BANULs and proanthocyanidin biosynthesis in Arabidopsis thaliana. - Plant J. 39: 366-380, 2005.

Bedon, F., Bomal, C., Caron, S., Levasseur, C., Boyle, B., Mansfield, S.D., Gershenzon, J., Grima-Pettenati, J., Séguin, A., MacKay, J.: Subgroup 4 R2R3-MYBs in conifer trees: gene family expansion and contribution to the isoprenoid- and flavonoid-oriented responses. - J. exp. Bot. 61: 3847-3864, 2010.

Bovy, A.G., De Vos, R., Kemper, M., Schijen, E., Pertejo, M.A., Muir, S., Collins, G., Robinson, S., Verhoeven, M., Hughes, S.: High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. - Plant Cell 14: 2509-2526, 2002.

Broun, P.: Transcriptional control of flavonoid biosynthesis: a complex network of conserved regulators involved in multiple aspects of differentiation in Arabidopsis. - Curr. Opin. Plant Biol. 8: 272-279, 2005.

Czemmel, S., Stracke, R., Weisshaar, B., Cordon, N., Harris, N.N., Walker, A.R., Robinson, S.P., Bogs, J.: The grapevine R2R3-MYB transcription factor VvMYBF1 regulate flavonol synthesis in developing grape berries. - Plant Physiol. 151: 1513-1530, 2009.

Debeaujon, I., Peeters, A.J., Léon-Kloosterziel, K.M., Koornneef, M.: The TRANSPARENT TESTA12 gene of Arabidopsis encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. - Plant Cell 13: 853-871, 2001.

Easley, R.V., Hellens, R.P., Putterill, J., Stevenson, D.E., Kutty-Aamma, S., Allan, A.C.: Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. - Plant J. 49: 414-427, 2007.

Finn, R.D., Tate, J., Mistry, J., Coggill, P.C., Sammut, S.J., Hotz, H.R., Ceric, G., Forslund, K., Eddy, S.R., Sonnhammer, E.L., Bateman, A.: The Pfam protein families database. - Nucl. Acids Res. 36: D281-D288, 2007.

Guan, C., Song, X., Ji, J., Li, X.Z., Jin, C., Li, J., Wang, G.: Salicylic acid treatment enhances expression of chalcone isomerase gene and accumulation of corresponding flavonoids.
during fruit maturation of *Lycium chinense*. - Eur. Food Res. Technol. 239: 857-865, 2014.

Gonzalez, A., Zhao, M., Leavitt, J.M., Lloyd, A.M.: Regulation of the anthocyanin biosynthetic pathway by the TIG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. - Plant J. 53: 430-440, 2008.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Ginrke, A., Rhind, N., Palma, F.D., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A.: Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. - Nat. Biotechnol. 29: 644-652, 2011.

Han, Y.J., Wang, X.H., Chen, W.C., Dong, M.F., Yuan, W.J., Shang, F.D.: Differential expression of carotenoid-related genes determines diversified carotenoid coloration in flower petal of *Osmanthus fragrans*. - Tree Genet. Genomes 10: 329-338, 2014a.

Han, S., Wang, C.W., Wang, W.L., Jiang, J.: Mitogen-activated protein kinase 6 controls root growth in *Arabidopsis* by modulating Ca2+-based Na+ flux in root cell under salt stress. - J. Plant Physiol. 171: 26-34, 2014b.

Han, Y.J., Chen, W.C., Yang, F.B., Wang, X.H., Dong, M.F., Zhou, P., Shang, F.D.: cDNA-AFLP analysis on 2 *Osmanthus fragrans* cultivars with different flower colour and molecular characteristics of *Os MYB* gene. - Trees 29: 931-940, 2015.

Han, Y.J., Liu, L.X., Dong, M.F., Yuan, W.J., Shang, F.D.: cDNA cloning of the phytoene synthase (PSY) and expression analysis of PSY and carotenoid cleavage dioxygenase genes in *Osmanthus fragrans*. - Biologia 68: 258-263, 2013.

Han, Y.J., Wang, H.Y., Wang, X.D., Li, K., Dong, M.F., Li, Y., Zhu, Q., Shang, F.D.: Mechanism of floral scent production in *Osmanthus fragrans* and the production and regulation of its key floral constituents, β-ionone and linalool. - Hort. Res. 6: 106, 2019.

Han, Y.J., Wu, M., Cao, L.Y., Shang, F.D.: Characterization of *OfWRKY3*, a transcription factor that positively regulates the carotenoid cleavage dioxygenase gene *OfCCD4* in *Osmanthus fragrans*. - Plant Mol. Biol. 91: 485-496, 2016.

Hao, G., Du, X., Zhao, F., Ji, H.: Fungal endophytes-induced abscisic acid is required for flavonoid accumulation in suspension cells of *Ginkgo biloba*. - Biotechnol. Lett. 32: 305-314, 2010.

Harris, M.A., Clark, J., Ireland, A., Foulger, R.: The Gene Ontology (GO) database and informatics resource. - Nucl. Acids Res. 32: D258-D261, 2004.

He, X.Y., Yuan, W.J., Dong, M.F., Han, Y.J., Shang, F.D.: The first genetic map in sweet osmanthus (*Osmanthus fragrans* Lour.) using specific locus amplified fragment sequencing. - Front. Plant Sci. 8: 1621, 2017.

Hernandez, J.M., Heine, G.F., Irani, N.G., Feller, A., Kim, M.G., Matulnik, T., Chandler, V.L., Grotewold, E.: Different mechanisms participate in the R-dependent activity of the R2R3-MYB transcription factor C1. - J. Biol. Chem. 279: 48205-48213, 2004.

Huse, S.M., Huber, J.A., Morrison, H.G., Sogin, M.L., Welch, D.M.: Accuracy and quality of massively parallel DNA pyrosequencing. - Genome Biol. 8: R143, 2007.

Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., Hattori, M.: The KEGG resource for deciphering the genome. - Nucleic Acids Res. 32: D254-D259, 2004.

Koes, R., Verweij, W., Quattrochio, F.: Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. - Trends Plant Sci. 10: 236-242, 2005.

Livak, K.J., Schmittgen, T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) method. - Methods 25: 402-408, 2001.

Love, M.I., Huber, W., Anders, S.: Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. - Genome Biol. 15: 550, 2014.

Margulis, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S.: Genome sequencing in microfabricated high-density picolitre reactors. - Nature 437: 376-380, 2005.

Mathews, H., Clendennen, S.K., Caldwell, C.G., Liu, X.L., Connors, K., Matheis, N., Schuster, D.K., Menasco, D.J., Wagoner, W., Lightner, J.: Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. - Plant Cell 15: 1689-1703, 2003.

Mendulkar, V.D., Vakil, M.M.A.: Elicitation of flavonoids by salicylic acid and *Penicillium expansum* in *Andropogon paniculata* (Burm. f.) Nees. cell culture. - Res. Biotechnol. 2: 1-9, 2013.

Mehrtens, K., Kranz, H., Bednarek, P., Weisshaar, B.: The *Arabidopsis* transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. - J. Plant Physiol. 138: 1083-1096, 2005.

Mondal, S.K., Roy, S.: Genome-wide sequential, evolutionary, organizational and expression analyses of phenylpropanoid biosynthesis associated MYB domain transcription factors in *Arabidopsis*. - J. Biomol. Struct. Dynamics 36: 1577-1601, 2017.

Morita, Y., Saitoh, M., Hoshino, A., Nitasaka, E., Iida, S.: Isolation of cDNAs for R2R3-MYB, bHLH and WDR transcriptional regulators and identification of c and ca mutations conferring white flowers in the Japanese morning glory. - Plant Cell Physiol. 47: 457-470, 2006.

Nakamura, N., Masako, F.M., Miyazaki, K., Tanaka, Y.: RNAi suppression of the anthocyanidin synthase gene in *Torenia hybrida* yields white flowers with higher frequency and better stability than antisense and sense suppression. - Plant Biotechnol. 23: 13-17, 2006.

Nakatsuka, T., Abe, Y., Kakizaki, Y., Yamanaka, S., Nishihara, M.: Production of red-flowered plants by genetic engineering of multiple flavonoid biosynthetic genes. - Plant Cell Rep. 26: 1951-1959, 2007.

Nesi, N., Jond, C., Debeaujon, I., Caboche, M., Lepiniec, L.: The *Arabidopsis TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. - Plant Cell 13: 2099-2114, 2001.

Nishihara, M., Nakatsuka, T., Yamanaka, S.: Flavonoid components and flower color change in transgenic tobacco plants by suppression of chalcone isomerase gene. - FEBS Lett. 579: 6074-6078, 2005.

Novacs, E., Drost, D.R., Farmerie, W.G., Pappas, G.J.Jr., Grattapaglia, D., Sederoff, R.R., Kirst, M.: High-throughput gene and SNP discovery in *Eucalyptus grandis*, an uncharacterized genome. - BMC Genomics 9: 312, 2008.

Peters, D.J., Constabel, C.P.: Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). - Plant J. 32: 701-712, 2002.

Qi, T., Xie, D.: The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate jasmonate-mediated anthocyanin accumulation and trichome initiation in *Arabidopsis thaliana*. - Plant Cell 23: 1795-1814, 2011.

Quattrochio, F., Verweij, W., Kroon, A., Spelt, C., Mol, J., Koes, R., Paih of *Petunia* is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix-loops-helix transcription factors of the anthocyanin pathway.
FLAVONOID BIOSYNTHESIS IN FLOWERS of OSMANTHUS FRAGRANS

- Plant Cell 18: 1274-1291, 2006.
Quattrocchio, F., Wing, J., Van der Woude, K., Souer, E., De Vetten, N., Mol, J., Koes, R.: Molecular analysis of the anthocyanin 2 gene of petunia and its role in the evolution of flower colour. - Plant Cell 11: 1433-1444, 1999.
Ramsay, N.A., Glover, B.J.: MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. - Trends Plant Sci. 10: 63-70, 2005.
Sonnhammer, E.L.L., Koonin, E.V.: Orthology, paralogy and proposed classification for paralog subtypes. - Trends Genet. 18: 619-620, 2003.
Spelt, C., Quattrocchio, F., Mol, J.N.M., Koes, R.: Anthocyanin1 of Petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. - Plant Cell 12: 1619-1632, 2000.
Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrten, F., Niehaus, K., Weisshaar, B.: Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. - Plant J. 50: 660-677, 2007.
Takos, A.M., Jaffé, F.W., Jacob, S.R., Bogs, J., Robinson, S.P., Walker, A.R.: Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. - Plant Physiol. 142: 1216-1232, 2006.
Tanaka, Y., Ohmiya, A.: Seeing is believing: engineering anthocyanin and carotenoid biosynthetic pathways. - Curr. Opin. Biotechnol. 19: 190-197, 2008.
Tanaka, Y., Sasaki, N., Ohmiya, A.: Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. - Plant J. 54: 733-749, 2010.
Tatusov, R.L., Fedorova, N.D., Jackson, J.D., Jacobs, A.R., Kiryutin, B., Koonin, E.V., Krylov, D.M., Mazumder, R., Mekhedov, S.L., Nikolskaya, A.N., Rao, B.S., Smirnov, S., Sverdlov, A.V., Vasudevan, S., Wolf, Y.I., Yin, J.J., Natale, D.A.: The COG database: an updated version includes eukaryotes. - BMC Bioinformatics 4: 41, 2003.
Tsuda, S., Fukui, Y., Nakamara, N., Katsumoto, Y., Sakakibara, K., Fukuchi-Mizutani, M., Ohira, K., Ueyama, Y., Ohkawa, H., Holton, T.A., Kusumi, T., Tanaka, Y.: Flower colour modification of Petunia hybrida commercial varieties by metabolic engineering. - Plant Biotechnol. 21: 377-386, 2004.
Vimolmangkang, S., Han, Y., Wei, G.C., Korban, S.S.: An apple MYB transcription factor, MdMYB3, is involved in regulation of anthocyanin biosynthesis and flower development. - BMC Plant Biol. 13: 176, 2013.
Wang, Z., Gerstein, M., Snyder, M.: RNA-Seq: a revolutionary tool for transcriptomics. - Nat. Rev. Genet. 10: 57-63, 2009.
Wei, W.L., Qi, X.Q., Wang, L.H., Zhang, Y.X., Hua, W., Li, D.H., Zhang, X.R.: Characterization of the sesame (Sesamum indicum L.) global transcriptome using Illumina paired-end sequencing and development of EST-SSR markers. - BMC Genomics 12: 451, 2011.
Winkel-Shirley, B.: Flavonoid biosynthesis. A colourful model for genetics, biochemistry, cell biology, and biotechnology. - Plant Physiol. 126: 485-493, 2001.
Xiang, Q.B., Liu, Y.L.: An Illustrated Monograph of the Sweet Osmanthus Varieties in China. - Zhejiang Science & Technology Press, Hangzhou 2007.
Zifkin, M., Jin, A., Ozga, J.A., Zaharia, L.I., Irina, L., Schermthainer, J.P., Gesell, A., Abrams, S.R., Kennedy, J.A., Constabel, C.P.: Gene expression and metabolite profiling of developing highbush blueberry fruit indicates transcriptional regulation of flavonoid metabolism and activation of abscisic acid metabolism. - Plant Physiol. 158: 200-224, 2012.
Zuker, A., Tzfira, T., Ben-Meir, H., Ovadis, M., Shklarman, E., Itzhaki, H., Forkamn, G., Martens, S., Nata-Sharrir, I., Weiss, D., Vainstein, A.: Modification of flower colour and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. - Mol Breed. 9: 33-41, 2002.