Identification of Candidate Anthocyanin-Related Genes by Transcriptomic Analysis of ‘Furongli’ Plum (Prunus salicina Lindl.) during Fruit Ripening Using RNA-Seq

Zhi-Zhen Fang, Dan-Rong Zhou, Xin-Fu Ye *, Cui-Cui Jiang and Shao-Lin Pan

Fruit Research Institute, Fujian Academy of Agricultural Sciences, Fuzhou, China

Anthocyanins are important pigments and are responsible for red coloration in plums. However, little is known about the molecular mechanisms underlying anthocyanin accumulation in plum fruits. In this study, the RNA-seq technique was used to analyze the transcriptomic changes during fruit ripening in the red-fleshed plum (Prunus salicina Lindl.) cultivar ‘Furongli’. Over 161 million high-quality reads were assembled into 52,093 unigenes and 49.4% of these were annotated using public databases. Of these, 25,681 unigenes had significant hits to the sequences in the NCBI Nr database, 17,203 unigenes showed significant similarity to known proteins in the Swiss-Prot database and 5816 and 8585 unigenes had significant similarity to existing sequences in the Kyoto Encyclopedia of Genes and Genomes and the Cluster of Orthologous Groups databases, respectively. A total of 3548 unigenes were differentially expressed during fruit ripening and 119 of these were annotated as involved in “biosynthesis of other secondary metabolites.” Biological pathway analysis and gene ontology term enrichment analysis revealed that 13 differentially expressed genes are involved in anthocyanin biosynthesis. Furthermore, transcription factors such as MYB and bHLH, which may control anthocyanin biosynthesis, were identified through coexpression analysis of transcription factors, and structural genes. Real-time qPCR analysis of candidate genes showed good correlation with the transcriptome data. These results contribute to our understanding of the molecular mechanisms underlying anthocyanin biosynthesis in plum flesh. The transcriptomic data generated in this study provide a basis for further studies of fruit ripening in plum.

Keywords: transcriptome, Prunus salicina Lindl., anthocyanin biosynthesis, fruit ripening, biosynthetic enzyme, transcription factor

INTRODUCTION

The plum is one of the traditional fruit trees in China and is widely distributed in the world (Carrasco et al., 2012). Its fruit is highly appreciated by consumers. Plums are rich in bioactive substances such as vitamin C, carotenoids, polyphenols, and anthocyanins (Valero et al., 2013) and have great health benefits (Hooshmand and Arjmandi, 2009; Lee et al., 2009; Shukitt-Hale et al., 2009; Johnson et al., 2011). Color is one of the most important determinants of fruit quality. As
a result of the influence of culture, Chinese people prefer red color. Anthocyanin accumulation is responsible for red coloration in plums (Cheng et al., 2015). Anthocyanins are widespread secondary metabolites that play an important role in the pigmentation of fruits. They are powerful antioxidants and naturally-occurring dietary anthocyanins are beneficial to human health (Nemie-Feyissa et al., 2015). Santhakumar et al. (2015) demonstrated that consumption of anthocyanin-rich plum juice attenuates thrombogenesis by reducing platelet activation/hypercoagulability and oxidative stress. It is therefore, desirable to increase the anthocyanin content in plums, especially in the flesh, through improved cultivation methods, postharvest handling, or breeding.

The anthocyanin biosynthetic pathway has been well studied in many plants. Structural genes in the anthocyanin pathway, including those encoding phenylalanine ammonialyase (PAL), cinnaamate-4-hydroxylase (C4H), 4-coumaroyl-CoA-ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3′H), flavonoid 3′-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase, dihydroflavonol 4-reductase (DFR), anthocyanidin synthase/leucoanthocyanidin dioxygenase (ANS/LDOX), and UDP-glucose: flavonoid 3-O-glucosyltransferase (UGFT), have been isolated and characterized in plants (Tanaka et al., 2008). After the synthesizing in the cytosol, anthocyanins must be transported to the vacuole to exhibit their brilliant colors (Winkel-Shirley, 2001). This process is mediated by proteins including glutathione S-transferase (GST), multidrug and toxic compound extrusion, and ATP-binding cassette transporters (Hu et al., 2016).

Anthocyanin biosynthesis is cooperatively regulated by transcriptional regulators including MYB proteins, basic helix-loop-helix (bHLH) proteins, and WD40 proteins (Lepiniec et al., 2006; Feller et al., 2011). These regulators form an MBW complex that binds to promoters and activates transcription of structural genes of the anthocyanin biosynthetic pathway (Rahim et al., 2014; Li et al., 2016). The role of MBW in anthocyanin biosynthesis has been elucidated in fruit trees such as grape (Kobayashi et al., 2002, 2004; Walker et al., 2007), Chinese bayberry (Niu et al., 2010; Liu et al., 2013a,b), mangosteen (Palapol et al., 2009), blood orange (Crifò et al., 2011; Butelli et al., 2012), kiwifruit (Fraser et al., 2013), litchi (Lai et al., 2014, 2016), sweet cherry (Shen et al., 2014), apple (Takos et al., 2006; Espley et al., 2007; Xie et al., 2012; Chagné et al., 2013; An et al., 2015), and peach (Rahim et al., 2014; Zhou et al., 2014; Tuan et al., 2015).

In addition, other regulatory factors also affect anthocyanin biosynthesis via interaction with MBW complex or by modulating the transcription of structural genes directly. MYBs that act as repressors of the anthocyanin pathway have been identified in several plants (Matsui et al., 2008; Salvatierra et al., 2013; Xu et al., 2013; Huang et al., 2014; Jun et al., 2015; Yoshida et al., 2015). Shin et al. (2007) found that PIF3 and HY5 regulated anthocyanin synthesis by binding directly to promoters of anthocyanin biosynthetic genes in Arabidopsis thaliana. The Arabidopsis COP1/SPA complex represses anthocyanin accumulation by degrading PAP1 and PAP2 proteins (Maier et al., 2013; Maier and Hoecker, 2014). Li et al. (2012b) also demonstrated that MdCOP1 negatively regulates accumulation of anthocyanin in apple peel by modulating the degradation of the MdMYB1 protein. ANAC078 has been shown to promote accumulation of anthocyanins by inducing the expression of flavonoid biosynthesis genes under high-light (Morishita et al., 2009). Recently, Zhou et al. (2015) found that NAC transcription factor BL, which controls the red-fleshed trait, interacts with PpNAC1 to form a heterodimer, and activate the transcription of PpMYB10.1 to induce anthocyanin accumulation. This process is repressed by a SQUAMOSA promoter-binding protein-like (SPL) transcription factor PpSPL1. SPLs have been shown to inhibit the expression of anthocyanin biosynthetic genes and negatively regulate anthocyanin accumulation through destabilization of MBW (Gou et al., 2011). MADS-box genes are reported to be involved in regulation of anthocyanin accumulation (Jaakola et al., 2010; Wu et al., 2013). Jasmornates induce anthocyanin biosynthesis through degradation of jasmonate-ZIM-domain (JAZ) proteins and the subsequent release of MBW (Qi et al., 2011). DELLAs proteins promote anthocyanin biosynthesis by sequestering MYB2 and JAZ proteins, which repress the activity of MBW, to release bHLH/MB subunits in Arabidopsis (Xie et al., 2016). Furthermore, epigenetic mechanisms also play important roles in anthocyanin biosynthesis (Wang et al., 2013; Zabala and Vodkin, 2014).

The ‘Furongli’ plum (Prunus salicina Lindl.), a red-skinned and red-fleshed cultivar, is native to Fujian, where it has been cultivated for more than 700 years. Fruit of ‘Furongli’ is popular for its attractive color, delicious taste, and health-promoting nutrients (Fang et al., 2014). The fruit can be eaten fresh and is used for the production of candied fruits. In recent years, RNA-seq-based transcriptome analysis has been extensively used for identification of functional genes in fruit trees. Rodamilans et al. (2014) analyzed the transcriptome changes in response to Plum pox virus infection in P. cerasifera. Jo et al. (2015) reported the leaf transcriptome assembly of two different P. salicina cultivars. More recently, González et al. (2016a) reported the fruit skin transcriptomes of two Japanese plum cultivars with different skin color and developed candidate EST–SSR markers for marker-assisted selection of fruit skin color in the Japanese plum (González et al., 2016b). In the present study, we analyzed the transcriptomic changes during the ripening of ‘Furongli’ plum fruits to identify candidate genes involved in the biosynthesis of anthocyanins. Based on the RNA-seq datasets generated, we identify several potential structural genes, and transcription factor genes related to anthocyanin biosynthesis. The transcriptomic data generated in this study provide a basis for further studies of fruit
Fang et al. Transcriptome of Plum during Fruit Ripening

ripening in plum, and identify candidate genes involved in sugar accumulation, organic acid degradation, fruit softening, and pigmentation.

MATERIALS AND METHODS

Plant Materials

All samples were collected from 5-year-old field grown ‘Furongli’ plum (Prunus salicina Lindl.) trees in an orchard in Yongtai County, Fujian Province, China. Fruit samples were harvested at 105, 115, 125, and 135 days after flowering (DAF) from three trees in 2014 (Figure 1A). Twenty representative fruits were sampled from each tree at each developmental stage and sliced. The sliced samples were combined and immediately frozen in liquid nitrogen and kept at −80°C until use.

Determination of Anthocyanin Content

Anthocyanin content was quantified as described by Niu et al. (2010). Briefly, approximately 3 g of sample was ground to a fine powder in liquid nitrogen and extracted with 20 mL extraction solution (0.05% HCl in methanol) at 4°C for 24 h. After centrifugation at 8000 × g for 20 min, the supernatant was transferred into a clean tube. One milliliter of supernatant and 4 mL of either buffer A (0.4 MKCl, adjusted to pH 1.0 with HCl) or buffer B (1.2 N citric acid, adjusted to pH 4.5 with Na2HPO4) were mixed and the absorbance at 510 and 700 nm (A510 and A700) measured for A and B buffers, respectively. The anthocyanin content was calculated according to Romero et al. (2008) using the following formula: TA = A × MW × 5 × 100 × V/e, where TA stands for total anthocyanin content (mg/100 g, as cyanidin-3-O-glucose equivalent), V for final volume (mL), and A = [A510 (pH 1.0) − A700 (pH 1.0)] − [A510 (pH 4.5) − A700 (pH 4.5)]. A molar absorptivity (e) of 26,900 and molecular weight (MW) of 449.2 were used according to Wrolstad et al. (1982). Three measurements were taken for each biological replicate.

RNA preparation, Library Construction, and RNA-Seq

Total RNA was extracted from each sample using a EZNA Plant RNA Kit (Omega Bio-tek). The concentration of RNA was quantified with a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, CA, USA), and RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Equal amounts of RNA from three samples at the same stage were mixed together. The RNA isolation, library construction, and RNA-seq were performed by staff at Beijing BioMarker Technologies (Beijing, China). cDNA libraries were constructed as described by Han et al. (2013). Poly-A mRNA was enriched using poly-T oligoattached magnetic beads, then broken into small pieces, and used as template for cDNA synthesis. First strand cDNA was synthesized using reverse transcriptase and random primers, followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. The cDNA libraries were sequenced on an Illumina HiSeq™ 2500.

De novo Transcriptome Assembly

Before assembly, the raw reads in FASTQ format were filtered using in-house Perl scripts to discarding the reads containing the sequencing adapters and low-quality reads with ambiguous “N” bases or in which more than 10% of bases had a Q ≤ 20. The left files (read1 files) from all libraries/samples were pooled into one large left.fq file, and right files (read2 files) into one large right.fq file. De novo assembly of the clean reads was performed using Trinity (Grabherr et al., 2011) with min_kmer_cov set to 2 by default and all other parameters set to their default values.

Functional Annotation and Classification

To annotate unigene sequences of ‘Furongli’ plums, blastx search (E < 10−5) was used to search against the NCBI nonredundant protein (nr), UniProt/Swiss-Prot, Gene Ontology (GO), Cluster of Orthologous Groups of proteins (COG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) and Swiss-Prot databases and retrieve protein functional annotations based
on sequence similarity. GO terms were assigned to the unigenes using Blast2GO (Conesa et al., 2005) with $E \leq 10^{-5}$. The distribution of the GO functional classifications of the unigenes was plotted using WEGO software (Ye et al., 2006).

**Unigene Differential Expression Analysis**

Reads from the four cDNA libraries were mapped to the assembled unigenes using Bowtie (Langmead et al., 2009). Unigene expression levels were quantified using fragments per kilobase of transcript per million mapped reads (FPKM). FPKM values were calculated using RSEM (Li and Dewey, 2011). For each sequenced library, the read counts were adjusted using edgeR (Robinson et al., 2010) with one normalization factor. Differential expression analysis of two samples was performed using the DESeq R package (Wang et al., 2010). Unigenes differentially expressed between two samples were screened using false discovery rate (FDR) < 0.01 and absolute log$_2$ fold changes ≥ 1 as the threshold.

**Prediction of Transcription Factors**

To identify transcription factors expressed during ripening of ‘Furongli’ plum fruit, predicted peptide sequences of all unigenes were searched against transcription factors in PlantTFDB 3.0 using the Transcription Factor Prediction module (http://planttfdb.cbi.pku.edu.cn/prediction.php) with default parameters.

**Correlation Analysis of Structural Genes and Transcription Factors**

Correlation analysis of anthocyanin structural genes and transcription factors was performed as described by Ye et al. (2015). To exclude false positives, we only selected unigenes with a FPKM value ≥10 in at least one of the four stages during fruit ripening. Coexpression analysis was carried out using the “CORREL” function in Microsoft Excel 2003 and confirmed using in-house Perl scripts and IBM SPSS statistics software.

**Real-Time Quantitative RT-PCR Analysis**

Nineteen candidate differentially expressed genes involved in anthocyanin biosynthesis were selected for validation by real-time quantitative RT-PCR (qRT-PCR). Total RNA from fruits samples was extracted using a modified CTAB method as described by Xuan et al. (2015). The primer sequences used for qRT-PCR are listed in Table S1. The cDNA was transcribed from 500 ng of total RNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara, Dalian, China). Quantitative RT-PCR was performed using the Eppendorf RealPlex® real-time PCR system (Hamburg, Germany) in a total volume of 20 µL in each well containing 10 µL of 2 × Sybr Premix Ex Taq™ II (Tli RNaseH Plus, TaKaRa), 1 µL of cDNA (in 1:10 dilution), and 0.4 µL 10 µM primers. Quantitative PCR conditions were 5 min at 95°C, followed by 40 cycles of 5 s at 95°C, 15 s at annealing temperatures listed in Table S1, and 30 s at 72°C, followed by 60–95°C melting curve detection. The actin gene was used as the reference. The expression levels were calculated as described by Fang et al. (2013). Three biological and three technical replicates were performed.

**RESULTS**

**Anthocyanin Accumulation during Ripening of ‘Furongli’ Plum Fruits**

As indicated in Figure 1A, the color of ‘Furongli’ plums changed from green to red during ripening and the flesh became pigmented before the skin. Anthocyanin accumulation is responsible for the red color of plums and the major anthocyanins in ‘Furongli’ plums are cyanidin 3-rutinoside and cyanidin 3-glucoside (Usenik et al., 2009; Zhang, 2009). The anthocyanin content of ‘Furongli’ plums increased from 0.13 mg/100 g FW to 7.0 mg/100 g FW as ripening proceeded (Figure 1B).

**RNA-Seq and De novo Transcriptome Assembly**

Four cDNA libraries were constructed from the total RNA of ‘Furongli’ plums at 105, 115, 125, and 135 DAF. These libraries were subjected to RNA-seq using an Illumina HiSeq2500, generating 41,555,484, 43,021,234, 40,991,934, and 37,023,409 raw 100-bp paired-end raw reads, respectively (Table 1). All of the raw reads are available in the NCBI SRA database (accession number SRP076001). After removing low-quality reads and trimming adapter sequences, 41,067,827, 42,872,666, 40,886,898 and 36,927,707 clean reads were obtained for the 105, 115, 125, and 135 DAF libraries, respectively.

Using Trinity, the clean reads from the four libraries were assembled into 100,711 transcripts with an average length of 1320 bp, and 52,093 unigenes with an average length of 872 bp were obtained (Table 1). Unigenes shorter than 500 bp accounted for 55.43% of the total unigenes and 26.77% of the unigenes (13,944) were longer than 1 kb (Figure 2). These results suggest that the quality of the unigene data was high enough for the following analyses.

**Functional Annotation and Classification**

To annotate the transcriptome of ‘Furongli’ plums, 52,093 unigenes were searched against five public databases (nr, UniProt/Swiss-Prot, GO, COG, and KEGG) with a cutoff E-value of 10$^{-5}$. The functional annotation results are listed in Table 2. In 49.4% of the unigenes (25,730) were identified. The remaining unigenes (50.6%) could not be annotated with known genes (Table 2), most likely because of omissions in the genomic information and the presence of short sequences. Only 947 (3.6%) of the unannotated unigenes were longer than 1000 bp, while 47.1% were shorter than 300 bp. The species distribution of the best match results in nr is indicated in Figure 3. The ‘Furongli’ plum unigenes showed the closest matches with Prunus persica (77.80%), followed by Fragaria vesca (5.32), Vitis vinifera (1.51), Zea mays (1.06), Theobroma cacao (0.71), Populus
TABLE 1 | Summary of sequencing and de novo assembly.

| Sequences | 105d | 115d | 125d | 135d |
|-----------|------|------|------|------|
| BEFORE TRIMMING |      |      |      |      |
| Total nucleotides (bp) | 8,394,207,768 | 8,690,289,268 | 8,280,370,668 | 7,478,728,618 |
| Number of raw reads | 41,555,484 | 43,021,234 | 40,991,934 | 37,023,409 |
| AFTER TRIMMING |      |      |      |      |
| Number of clean reads | 41,067,827 | 42,872,666 | 40,886,898 | 36,927,707 |
| GC content (%) | 46.96 | 47.07 | 47.03 | 46.54 |
| Q30 percentage (%) | 86.11 | 86.02 | 86.46 | 87.49 |
| AFTER ASSEMBLY |      |      |      |      |
| Number of transcripts of combined data | 100,711 |      |      |      |
| Number of unigenes of combined data | 52,093 |      |      |      |
| Total nucleotides (nt) of transcripts (bp) | 132,974,754 |      |      |      |
| Total nucleotides (nt) of unigenes (bp) | 45,459,242 |      |      |      |
| Mean length of transcripts (bp) | 1320 |      |      |      |
| Mean length of unigenes (bp) | 872 |      |      |      |
| N50 of unigenes (bp) | 1675 |      |      |      |

FIGURE 2 | Sequence length distribution of the unigenes in ‘Furongli’ plum fruit transcriptomes. The x-axis indicates unigene length interval from 200 bp to >3000 bp. The y-axis indicates the number of unigenes of each given sequence length.

trichocarpa (0.56), Hordeum vulgare (0.48), Ricinus communis (0.46), Medicago truncatula (0.38), and Glycine max (0.37%).

The ‘Furongli’ plum unigenes were searched against the GO database to classify standardized gene functions. At least one GO term was assigned to 18,623 of the unigenes (Table 2). Unigenes were assigned to three main GO categories, including biological process category, cellular component category, and molecular function category, and 58 subcategories shown in Figure 4. The terms “cell,” “cell part,” and “organelle” were dominant in the cellular component category, the term “binding” and “catalytic activity” was dominant in the molecular function category, and the terms “metabolic process” and “cellular process” were dominant in the biological process category.

The completeness of the plum transcriptome library and the validity of the annotations were further evaluated by COG annotation. Out of the 25,730 annotated unigenes, 8585 (33.37%) were clustered into 24 COG categories (Figure 5). The cluster for “general functional prediction only” (2260, 26.32%) represented the largest group, followed by “transcription, ribosomal structure and biogenesis” (1095, 12.75%), “replication, recombination, and repair” (1074, 12.51%), “transcription” (1038, 12.09%), “Signal transduction mechanisms” (887, 10.33%), “posttranslational modification, protein turnover, chaperones” (840, 9.78%), “carbohydrate transport and metabolism” (652, 7.59%), “amino acid transport and metabolism” (553, 6.44%), and “energy production and metabolism” (516, 6.01%). “cell motility” (9, 0.1%) and “nuclear structure” (1, 0.01%) represented the smallest groups. No unigene was assigned to “extracellular structures.”

To better understand biological pathways involved in ripening of ‘Furongli’ plum fruits, all unigenes were searched against...
Between 115 and 125 DAF, as well as 1004 differentially expressed unigenes (778 upregulated and 886 downregulated) identified between 105 and 115 DAF, with 1674 differentially unigenes (304 upregulated and 203 downregulated) were detected at a single stage of development. Of these stage-specific unigenes (32,926, 63.21%) were expressed in all four stages and 135 DAF. The unigene c8193.graph_c0 (CHI) was assigned to “anthocyanin-containing compound biosynthetic process.” The unigene c25083.graph_c0 (flavonoid 3′-monooxygenase) was predicted to have flavonoid 3′, 5′-hydroxylase activity (GO:0033772). Furthermore, a GST (c29416.graph_c0), with best match to AtGSTF12 (AT5G17220), also showed differential expression. As shown in Table 3, the expression of these 13 unigenes was significantly upregulated during fruit maturation.

To identify unigenes involved in anthocyanin biosynthesis, KEGG functional enrichment was analyzed to characterize the functions of differentially expressed unigenes (Table S3). A total of 11 unigenes encoding enzymes, including PAL (c38988.graph_c0), C4H (c23939.graph_c0), 4CL (c30378.graph_c0), CHS (c37054.graph_c0), CHI (c28749.graph_c0), F3H (c23888.graph_c0), F3′H (c38186.graph_c0), DFR (c24831.graph_c0), ANS/LDOX (c29583.graph_c0), and UFGT (c19095.graph_c0), were assigned to the anthocyanin biosynthetic pathway based on KEGG (Table 3; Table S3). GO annotation was carried out to further identify anthocyanin-associated unigenes without annotation information in KEGG database (Table S4). The unigene c8193.graph_c0 (CHI) was assigned to “anthocyanin-containing compound biosynthetic process.” The unigene c25083.graph_c0 (flavonoid 3′-monooxygenase) was predicted to have flavonoid 3′, 5′-hydroxylase activity (GO:0033772). Furthermore, a GST (c29416.graph_c0), with best match to AtGSTF12 (AT5G17220), also showed differential expression. As shown in Table 3, the expression of these 13 unigenes was significantly upregulated during fruit maturation.

Transcription factors play important roles in the regulation of anthocyanin biosynthesis. In total, 791 unigenes (Table S5) were predicted to encode transcription factors from 55 different families (Table S6) and 147 of them were differentially expressed (Table S7). To identify transcription factors that were coexpressed with the candidate enzymatic genes involved in anthocyanin biosynthesis, a transcription abundance correlation analysis was carried out between the differentially expressed transcription factors and structural genes from the anthocyanin biosynthetic pathway. This identified 37 transcription factors whose expression levels were highly correlated with those of the candidate structural genes (Table 4; Table S8). Of these, 22 showed a significant correlation with five or more structural genes from the anthocyanin biosynthetic pathway. The identified transcription factors included homologs of Arabidopsis transcription factors that are implicated in regulating anthocyanin biosynthesis, such as MYB, bHLH, and NAC (Table 3).

A total of 37 MYBs were differentially expressed during ripening of ‘Furongli’ plums and three of them (c39005.graph_c0, c29499.graph_c0 and c32850.graph_c0) were associated with the anthocyanin biosynthetic pathway (Table 4; Table S8). The unigene c39005.graph_c0 (homologous to AtMYB113) was upregulated, while c29499.graph_c0 (homologous to AtMYB73) and c32850.graph_c0 (homologous to AtMYB102) were downregulated. The expression level of the homolog of AtMYBD (c28480.graph_c0) also increased. However, it was correlated with none of the structural genes. Seven of the differentially expressed transcription factors annotated as bHLH showed...
a significant correlation with anthocyanin biosynthetic genes (Table 4; Table S8). Only two of the bHLH genes (c7988.graph_c0 and c18575.graph_c1) showed a positive correlation with the expression of structural genes, while most of them were negatively correlated with that of structural genes involved in the anthocyanin biosynthetic pathway. In addition, a plum bHLH (c36695.graph_c0, log2 fold change <1.0), which is the best BLAST match to *Arabidopsis* AtbHLH42, was significantly correlated with seven anthocyanin biosynthetic structural genes (Table S8). WD40 encoding unigenes (c28377.graph_c0 and c10590.graph_c1), which are the homolog of *AtTTG1*, did not show differential expression during the ripening process. Apart from the MBW components, other differentially expressed transcription factors, such as NAC, were also found to be potentially related to the anthocyanin pathway. Four plum NAC genes (c19087.graph_c0, c19209.graph_c0, c27539.graph_c0, and c37766.graph_c1) were significantly correlated with structural genes (Table 4; Table S8). The unigenes c27539.graph_c0 (a
homolog of AtNAC100) and c19209.graph_c0 were upregulated during plum fruit ripening. Peach homolog of AtNAC100 have been reported to be involved in the regulation of anthocyanin accumulation in fruit flesh. GO annotation results indicated that c19209.graph_c0 is involved in “biological process: positive regulation of flavonoid biosynthetic process” (GO:0009963).

We further analyzed the expression profiles of 19 candidate unigenes (13 structural genes and six transcription factors) involved in anthocyanin biosynthesis using qRT-PCR. The results
indicated that there is a good correlation between RNA-seq data and qPCR data for most of the genes (Figure 9).

DISCUSSION

In this study, we constructed a transcriptome of ‘Furongli’ plums during fruit maturation. In total, 52,093 unigenes were assembled with a mean length of 872 bp, which is comparable to 944 bp for sweet cherry (P. avium L.) (Wei et al., 2015). A large quantity of genomic data is available for many rosaceous plants, but only 49.4% of the plum unigenes were annotated to public databases (nr, Swiss-Prot, GO, COG, and KEGG). This means that more than half of the unigenes have no significant known homologs. The low rate of annotated unigenes could be a result of limitations in the genomic information available for P. salicina Lindl. as is the case in other non-model plant species (Yates et al., 2014; Wei et al., 2015; Wu et al., 2015). As expected, 77.80% of the unigenes annotated using nr show significant similarity to P. persica transcripts. The unannotated unigenes could be plum specific genes with novel functions. The transcriptome of ‘Furongli’ plums will serve as an important datasets for studying plum ripening processes such as sugar accumulation, organic acid degradation, fruit softening, and pigmentation.

Anthocyanin-rich plums are of great interest for their implications in human health (Santhakumar et al., 2015). The main objective of this study was to identify genes involved in anthocyanin biosynthesis in plums. RNA-seq-based comparative transcriptome analysis has been shown to be an efficient strategy for the investigation of genes involved in anthocyanin biosynthesis in several plants, such as kiwifruit (Li et al., 2015a), sweet cherry (Wei et al., 2015), zoysiagrass (Ahn et al., 2015), anthurium (Li et al., 2015b), and potato (Liu et al., 2015). In the later ripening stages, ‘Furongli’ plums accumulate anthocyanins rapidly (Figure 1). The expression of anthocyanin biosynthetic genes has been shown to be correlated with fruit anthocyanin content in Rosaceae such as apple (Feng et al., 2013, 2014; Vimolmangkang et al., 2014), pear (Li et al., 2012a; Yang et al., 2015), sweet cherry (Wei et al., 2015), strawberry (Xu et al., 2014), and plum (Cheng et al., 2015). In the present study, changes in gene expression between different stages of ripening were analyzed to identify differentially expressed genes implicated in anthocyanin biosynthesis, including PAL, C4H, 4CL, CHS, CHI, F3H, F3′H, DFR, ANS/LDOX, UFGT, and GST, were significantly upregulated in the late stages of fruit maturation (Table 3; Figure 9).

Anthocyanin biosynthesis is regulated by several well-studied transcription factors such as MYB, bHLH, and WD40 (Gonzalez et al., 2008). MYB transcription factors have been reported to play a pivotal role in anthocyanin biosynthesis regulation in several fruit trees (Chagné et al., 2013; Ravaglia et al., 2013; Umemura et al., 2013; Lai et al., 2014; Shen et al., 2014; Tuan et al., 2015; Zhai et al., 2015; Jin et al., 2016). Lin-Wang et al. (2010) demonstrated that R2R3 MYBs are highly conserved in rosaceous plants and MYBs from European plum and cherry plum are able to induce the anthocyanin accumulation in tobacco. Gu et al. (2015) proposed that constitutive activation of PcMYB10.6 is responsible for red pigmentation in purple-leaf plum. Cheng et al. (2015) indicated that PsMYB10 was

### Table 3 | Expression profiles of anthocyanin biosynthesis genes in ‘Furongli’ plums.

| Gene name | Unigene ID | Gene length | FPKM |
|-----------|------------|-------------|------|
| PAL       | c38398.graph_c0 | 2664        | 10.51 |
| C4H       | c23939.graph_c0 | 2195        | 45.98 |
| 4CL       | c30378.graph_c0 | 2101        | 22.54 |
| CHS       | c37054.graph_c0 | 1946        | 5.46  |
| CHI       | c28749.graph_c0 | 1168        | 63.12 |
| F3H       | c28888.graph_c0 | 1588        | 60.75 |
| F3′H      | c38186.graph_c0 | 2020        | 253.50|
| DFR       | c25083.graph_c0 | 2408        | 23.81 |
| GST       | c26583.graph_c0 | 1673        | 245.80|
| UFGT      | c19096.graph_c0 | 1619        | 23.47 |
| MYB       | c39005.graph_c0 | 1496        | 8.84  |
| MYBD      | c24831.graph_c0 | 1476        | 2.55  |
| bHLH      | c25083.graph_c0 | 2408        | 23.81 |
| NAC       | c27539.graph_c0 | 1674        | 4.02  |

Table 3 | Expression profiles of anthocyanin biosynthesis genes in ‘Furongli’ plums.

- **Gene name**: Name of the gene.
- **Unigene ID**: Identification number of the unigene.
- **Gene length**: Length of the gene in base pairs.
- **FPKM**: FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values for different stages of ripening (105 DAF, 115 DAF, 125 DAF, 135 DAF).
Table 4: Correlation analysis of structural genes involved in anthocyanin metabolism and transcription factors.

| Gene ID          | FPKM max | FPKM min | Description for the best hit in A. thaliana | Number of correlations |
|------------------|----------|----------|---------------------------------------------|------------------------|
| c39005.graph_c0  | 26.56    | 6.84     | AtMYB113                                    | 6                      |
| c29499.graph_c0  | 40.87    | 8.06     | AtMYB73                                     | 7                      |
| c32850.graph_c0  | 26.41    | 8.02     | AtMYB102                                    | 3                      |
| c7988.graph_c0   | 30.47    | 13.12    | CRY2-interacting bHLH3                      | 6                      |
| c18575.graph_c1  | 15.67    | 4.22     | AtbHLH135                                   | 1                      |
| c19069.graph_c1  | 135.85   | 28.98    | Transcription factor bHLH36                 | 4                      |
| c19862.graph_c0  | 42.35    | 7.56     | AtbHLH95                                    | 7                      |
| c33382.graph_c0  | 14.90    | 2.04     | AtbHLH14                                    | 6                      |
| c36134.graph_c1  | 37.33    | 16.77    | AtbHLH130                                   | 2                      |
| c38825.graph_c0  | 44.87    | 14.34    | Zinc finger protein ZAT17                   | 2                      |
| c33970.graph_c0  | 38.21    | 16.16    | Zinc finger protein JACKDAW                 | 5                      |
| c19067.graph_c0  | 342.48   | 92.52    | ANAC2                                       | 3                      |
| c19209.graph_c0  | 36.99    | 16.10    | NAC domain containing protein 83            | 4                      |
| c27539.graph_c0  | 18.54    | 4.02     | NAC domain-containing protein 100           | 6                      |
| c37766.graph_c1  | 16.22    | 7.19     | NAC014                                       | 5                      |
| c24992.graph_c1  | 98.05    | 45.08    | Ethylene response factor 61                 | 3                      |
| c8431.graph_c0   | 301.81   | 132.51   | Ethylene responsive element binding factor 5| 1                      |
| c18924.graph_c0  | 424.36   | 180.10   | Ethylene-responsive transcription factor      | 3                      |
| c24113.graph_c0  | 20.46    | 5.10     | Ethylene-responsive element binding factor 13| 8                      |
| c28635.graph_c0  | 71.57    | 10.92    | FYF up-regulating 321 factor 1               | 6                      |
| c28868.graph_c0  | 140.98   | 50.99    | Ethylene responsive element binding factor 1| 4                      |
| c39002.graph_c0  | 24.73    | 4.64     | C-repeat-binding factor 4                    | 8                      |
| c34188.graph_c0  | 33.98    | 7.08     | Cytokinin response factor 4                  | 6                      |
| c30911.graph_c0  | 17.26    | 7.99     | WRKY DNA-binding protein 4                   | 3                      |
| c23690.graph_c0  | 13.81    | 5.05     | WRKY transcription factor 29                 | 9                      |
| c5911.graph_c0   | 11.66    | 3.24     | bZIP transcription factor family protein TGA7| 7                      |
| c14559.graph_c0  | 48.74    | 8.51     | basic leucine zipper 9                      | 9                      |
| c18932.graph_c0  | 402.53   | 106.55   | basic leucine-zipper 44                     | 8                      |
| c38252.graph_c0  | 370.27   | 89.02    | basic leucine-zipper 44                     | 6                      |
| c38790.graph_c0  | 65.16    | 10.19    | basic leucine-zipper 6                      | 6                      |
| c33394.graph_c0  | 13.24    | 5.35     | AtC3H49                                      | 3                      |
| c35600.graph_c0  | 31.29    | 13.06    | AFN1                                         | 8                      |
| c19054.graph_c0  | 179.42   | 84.98    | Homeobox-leucine zipper protein HAT5         | 5                      |
| c34316.graph_c0  | 26.17    | 6.18     | Homeobox-leucine zipper protein ATHB-13      | 6                      |
| c38928.graph_c0  | 23.59    | 10.91    | Auxin response factor 17                     | 5                      |
| c33782.graph_c0  | 10.53    | 4.47     | Zinc finger protein constans-like 2          | 3                      |
| c28312.graph_c0  | 87.28    | 32.39    | AP2/ERF and B3 domain-containing transcription repressor TEM1 | 4 |
assays (Liu et al., 2013b; Rahim et al., 2014; Feng et al., 2015; Starkevič et al., 2015; Wei et al., 2015; Lai et al., 2016). Our results indicated that c36695.graph_c0, which is highly homologous to AtTT8 (AT4G09820), accumulates to higher levels in late stages of ripening and shows significant correlation with anthocyanin biosynthetic genes. Conversely, the expression of c33382.graph_c0 was repressed as ripening proceeded. The unigene c33382.graph_c0 is a homolog of AtbHLH14.
(AT4G00870), which belongs to bHLH subgroup IIId. Song et al. (2013) demonstrated that Arabidopsis lines overexpressing bHLH17 showed jasmonate-induced anthocyanin accumulation. NAC proteins have also been reported to be involved in anthocyanin synthesis in Arabidopsis (Morishita et al., 2009). Recently, a peach homolog of AtNAC100 was shown to be responsible for regulation of anthocyanin accumulation in flesh (Zhou et al., 2015). Our results indicated that a plum homolog of AtNAC100 (c27539.graph_c0) is upregulated and positively correlated with anthocyanin biosynthetic genes. It should be noted that coexpression analysis usually requires a large sample size and the small sample size in our study will reduce the reliability of our results. However, relatively small numbers of samples have recently been used to analyze the correlation of structural genes and regulators involved in biological processes, such as flavonoid biosynthesis (Zhai et al., 2015) and fruit ripening (Wu et al., 2016). The exact roles of these candidate transcription factor should be investigated in further studies.

In the current study, we used RNA-seq to analyze changes in the transcriptome during ripening of ‘Furongli’ plums. We generated 52,093 unigenes and over 50% of them were not annotated to public databases. Unigenes differentially expressed during fruit ripening were identified. Candidate genes encoding anthocyanin biosynthetic enzymes and transcription factors involved in anthocyanin biosynthesis were identified using functional annotation and coexpression analysis of differentially expressed genes. The expression patterns of some candidate genes encode anthocyanin biosynthetic enzymes and transcription factors were further validated by qRT-PCR. This provides an important datasets for studying fruit ripening processes, especially anthocyanin biosynthesis, in plums. Further studies are needed to determine whether the identified candidate genes are related to anthocyanin biosynthesis in plum.

AUTHOR CONTRIBUTIONS

This study was conceived by ZF and XY. The plant material preparation were carried out by ZF and SP. ZF, XY, CJ, and DZ analyzed the RNA-seq data. ZF, CJ, DZ, and SP performed the laboratory experiments and analyses. ZF drafted the manuscript. XY revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was funded by the Basic Scientific Research Funds of Public Welfare Scientific Research Institutes of Fujian Province (2014R1014-13), the Major Projects of Science and Technology Project of Fujian Province (2013N0002-1), the Youth Talent Plan of Fujian Academy of Agricultural Sciences (YC2015-9) and the Natural Science Foundation of Fujian Province (2016J01123 and 2015J05058).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01338

REFERENCES

Ahn, J. H., Kim, J. S., Kim, S., Soh, H. Y., Shin, H., Jang, H., et al. (2015). De novo transcriptome analysis to identify anthocyanin biosynthesis genes responsible for tissue-specific pigmentation in Zosysia grass (Zosysia japonica Steud.). PLoS ONE 10:e0124497. doi: 10.1371/journal.pone.0124497

An, X., Tian, Y., Chen, K., Liu, X., Liu, D., Xie, X., et al. (2015). MdMYB9 and MdMYB11 are involved in the regulation of the JA-induced biosynthesis of anthocyanin and proanthocyanidin in apples. Plant Cell Physiol. 56, 650–662. doi: 10.1093/pcp/pct205

Butelli, E., Licciardello, C., Zhang, Y., Liu, J., Mackay, S., Bailey, P., et al. (2012). Retrotransposons control fruit-specific, cold-dependent accumulation of anthocyanins in blood oranges. Plant Cell 24, 1242–1255. doi:10.1105/tpc.111.095232

Carrasco, B., Diaz, C., Moya, M., Gebauer, M., and Garcia-Gonzalez, R. (2012). Genetic characterization of Japanese plum cultivars (Prunus salicina) using SSR and ISSR molecular markers. Cien. Cien. Agrono. 39, 533–543. doi: 10.1067/S0718-16202012000300012

Cavallini, E., Matus, J. T., Finizio, L., Zenoni, S., Loyola, R., Guzzo, F., et al. (2015). The phenylpropanoid pathway is controlled at different branches by a set of two R2R3-MYB C2 repressors in grapevine. Plant Physiol. 167, 1448–1470. doi: 10.1104/pp.15.025172

Chagné, D., Lin-Wang, K., Espley, R. V., Volz, R. K., How, N. M., Rouse, S., et al. (2013). An ancient duplication of apple MYB transcription factors is responsible for novel red fruit-flesh phenotypes. Plant Physiol. 161, 225–239. doi: 10.1104/pp.112.206771

Cheng, Y., Liu, L., Yuan, C., and Guan, J. (2015). Molecular characterization of ethylene-regulated anthocyanin biosynthesis in plums during fruit ripening. Plant Mol. Biol. Rep. 34, 777–785. doi: 10.1007/s11105-015-0963-x

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676. doi: 10.1093/bioinformatics/bti610

Crifo, T., Petrone, G., Lo Cicero, L., and Lo Piero, A. R. (2011). Short cold storage enhances the anthocyanin contents and level of transcripts related to their biosynthesis in blood oranges. J. Agr. Food. Chem. 60, 476–481. doi:10.1021/jf103891e

Dubos, C., Le Gourrierec, J., Baudry, A., Huep, G., Lanet, E., Debeaujon, I., et al. (2008). MYBL2 is a new regulator of flavonoid biosynthesis in Arabidopsis thaliana. Plant J. 55, 940–953. doi: 10.1111/j.1365-313X.2008.03564.x

Espley, R. V., Hellens, R. P., Putterill, J., Stevenson, D. E., Kutty-Amma, S., and Allan, A. C. (2007). Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J. 49, 414–427. doi: 10.1111/j.1365-313X.2006.02964.x

Fang, Z., Zhou, D., Liao, R., Jiang, C., Pan, S., and Ye, X. (2014). ISSR analysis of Furong plums from Ninggang and Fujian. South China Fruits 43, 12–14.

Fang, Z. Z., Lai, C., C., Zhang, Y. L., Lin, Y. L., and Lai, Z. X. (2013). Identification of a PTC-containing DRLan transcript and its differential expression during somatic embryogenesis in Docucassa longan. Gene 529, 37–44. doi: 10.1016/j.gene.2013.07.091

Feller, A., Machemer, K., Braun, E. L., and Grotewold, E. (2011). Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. Plant J. 66, 94–116. doi:10.1111/j.1365-313X.2010.04459.x

Feng, F., Li, M., Ma, F., and Cheng, L. (2013). Phenylpropanoid metabolites and expression of key genes involved in anthocyanin biosynthesis in the shaded peel of apple fruit in response to sun exposure. Plant Physiol. Biochem. 69, 54–61. doi: 10.1016/j.plaphy.2013.04.020

Feng, F., Li, M., Ma, F., and Cheng, L. (2014). The effects of bagging and debagging on external fruit quality, metabolites, and the expression of anthocyanin
biothesynthetic genes in 'Jonagold' apple (Malus domestica Borkh). Sci. Hort. 165, 123–131. doi: /10.1016/j.scienta.2013.11.008

Feng, S., Sun, S., Chen, X., Wu, S., Wang, D., and Chen, X. (2015). PyMYB10 and PyMYB10.1 interact with bHLH to enhance anthocyanin accumulation in pears. PLoS ONE 10(1):e014212. doi: 10.1371/journal.pone.014212

Fischer, L., Seal, A., Montemurri, M., Morishige, T., Tsang, G., Datson, P., et al. (2013). An R2R3 MYB transcription factor determines red petal colour in an Actinidia (kiwifruit) hybrid population. BMC Genomics 14:28. doi: 10.1186/1471-2164-14-28

Gonzalez, A., Zhao, M., Leavitt, J. M., and Lloyd, A. M. (2008). Regulation of the anthocyanin biosynthetic pathway by the TTF1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. Plant J. 53, 814–827. doi: 10.1111/j.1365-313X.2007.03373.x

González, M., Salazar, E., Castillo, J., Morales, P., Mura-Jornet, I., Maldonado, J., et al. (2016). Full-length transcriptome assembly from RNA-Seq data with or without a reference genome. Nat. Biotechnol. 34, 644–652. doi: 10.1038/nbt.1388

Gu, C., Liao, L., Zhou, H., Wang, L., Deng, X., and Han, Y. (2015). Constitutive activation of an anthocyanin regulatory gene PcMYB10.6 is related to red coloration in purple-fruitage plum. PLoS ONE 10(4):e0135159. doi: 10.1371/journal.pone.0135159

Han, X.-J., Wang, Y.-D., Chen, Y.-C., Lin, L.-Y., and Wu, Q.-K. (2013). Transcriptome sequencing and expression analysis of terpenoid biosynthesis genes in Litchi chinensis. PLoS ONE 8(8):e73626. doi: 10.1371/journal.pone.0073626

Hu, B., Zhao, J., Lai, B., Qin, Y., Wang, H., and Hu, G. (2016). LcGST4 is an anthocyanin-related glutathione S-transferase gene in Litchi chinensis Sonn. Plant Cell Rep. 35, 831–841. doi: 10.1007/s00299-015-1924-4

Huang, Y. F., Viale, S., Guiraud, J. L., Torregrosa, L., Bertrand, Y., Cheynier, V., et al. (2014). A negative MYB regulator of proanthocyanidin accumulation, identified through expression quantitative locus mapping in the grape berry. New Phytol. 201, 795–809. doi: 10.1111/nph.12553

Jaskola, L., Poole, M., Jones, M. O., Kämäräinen-Karppinen, T., Koskimäki, J. J., Kobayashi, S., Ishimaru, M., Hiraoka, K., and Honda, C. (2002). Myb-related genes of the Kyoho grape (Vitis labruscana) regulate anthocyanin biosynthesis. Planta 215, 924–933. doi: 10.1007/s00425-002-0830-5

Lai, B., Du, L., Liu, R., Hu, B., Su, W., Qin, Y., et al. (2016). Two LcbHHL transcription factors interacting with LeMYB1 in regulating late structural gene of anthocyanin biosynthesis in Nictiana and Litchi chinensis during anthocyanin accumulation. Front. Plant Sci. 7:166. doi: 10.3389/fpls.2016.00166

Lin-Wang, K., Micheletti, D., Palmer, J., Volz, R., Lozano, L., Elespey, R., et al. (2012a). Differential expression of anthocyanin biosynthetic genes and transcription factor PcMYB10 in pears (Pyrus communis L.). PLoS ONE 7(4):e34670. doi: 10.1371/journal.pone.0034670

Li, P., Chen, B., Zhang, G., Chen, L., Dong, Q., Wen, J., et al. (2016). Regulation of anthocyanin and proanthocyanidin biosynthesis by Medicago truncatula bHLH transcription factor MfT88. New Phytol. 210, 905–921. doi: 10.1111/nph.13816

Li, W., Liu, Y., Zeng, S., Xiao, G., Wang, G., Yang, Y., et al. (2015a). Gene expression profiling of development and anthocyanin accumulation in kiwifruit (Actinidia chinensis) based on transcriptome sequencing. PLoS ONE 10(9):e0136439. doi: 10.1371/journal.pone.0136439

Li, Y., Zhao, K., Zhao, C., Zhao, X., Zhang, H., Shu, H., et al. (2012b). MdCoPI ubiquitin E3 ligases interact with MdMYB1 to regulate light-induced anthocyanin biosynthesis and red fruit coloration in apple. Plant Physiol. 160, 1011–1022. doi: 10.1104/pp.111.199703

Li, Z., Wang, J., Zhang, X., and Xu, L. (2015b). Comparative transcriptome analysis of Anthurium “Albama” and its anthocyanin-loss mutant. PLoS ONE 10(11):e0141907. doi: 10.1371/journal.pone.0141907

Lin-Wang, K., Boliho, K., Grafton, K., Kortstee, A., Karunaratnam, S., McGhee, T., et al. (2010). An R2R3 MYB transcription factor associated with regulation of the MYB transcription factors PAP1 and PAP2 involved in anthocyanin accumulation under low light and high light conditions. Plant J. 64, 924–934. doi: 10.1111/j.1365-313X.2010.04336.x

Liu, Y., Xue, X., Zao, X., Zhao, X., Zhu, X., and Chen, K. (2013a). The MrWD40-1 gene of Chinese bayberry (Myrica rubra) interacts with MYB and bHLH to enhance anthocyanin accumulation. Plant Mol. Biol. Rep. 31, 1474–1484. doi: 10.1007/s11105-013-0621-0

Liu, Y., Xue, X., Zao, X., Zhu, X., Zhao, X., and Chen, K. (2013b). The role of MrbHLH1 and MrbMYB1 in regulating anthocyanin biosynthetic genes in tobacco and Chinese bayberry (Myrica rubra) during anthocyanin biosynthesis. Plant Cell Tissue Organ Cult. 115, 285–298. doi: 10.1007/s11210-013-0361-8

Maier, A., Schrader, A., Kokkelink, L., Falke, C., Welter, B., Iniesta, E., et al. (2013). Light and the E3 ubiquitin ligase COPII/SPA control the protein stability of the MYB transcription factors PAPI and PAP2 involved in anthocyanin accumulation in Arabidopsis. Plant J. 74, 638–651. doi: 10.1111/tjp.12153

Frontiers in Plant Science | www.frontiersin.org
13 August 2016 | Volume 7 | Article 1338

Fang et al. Transcriptome of Plum during Fruit Ripening
Fang et al. Transcriptome of Plum during Fruit Ripening

Matsui, K., Umemura, Y., and Ohme-Takagi, M. (2008). AtMYB21, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in Arabidopsis. Plant J. 55, 954–967. doi: 10.1111/j.1365-313X.2008.03565.x

Morishita, T., Kojima, Y., Maruta, T., Nishizawa-Yokoi, A., Yabuta, Y., and Shigeoka, S. (2009). Arabidopsis NAC transcription factor, ANAC078, regulates flavonoid biosynthesis under high-light. Plant Cell Physiol. 50, 2210–2222. doi: 10.1093/pcp/pct159

Nemie-Feyissa, D., Heidari, B., Blaise, M., and Lillo, C. (2015). Analysis of interactions between heterologously produced bHLH and MYB proteins that regulate anthocyanin biosynthesis: quantitative interaction kinetics by Microscale Thermophoresis. Phytochemistry 111, 21–26. doi: 10.1016/j.phytochem.2015.01.004

Nguyen, N. H., Jeong, C. Y., Kang, G.-H., Yoo, S.-D., Hong, S.-W., and Lee, K. (2011). The Arabidopsis proteins that regulate anthocyanin biosynthesis: quantitative interaction kinetics by Microscale Thermophoresis. Phytochemistry 111, 21–26. doi: 10.1016/j.phytochem.2015.01.004

Qiu, T., Song, S., Ren, Q., Wu, D., Huang, H., and Chen, Y., et al. (2013). The bHLH transcription factors family in grapevine suppresses the anthocyanin biosynthetic gene promoters in Prunus domestica L. Plant Physiol. 165, 522–530. doi: 10.1093/plantphys/kip036

Santhakumar, A. B., Kundur, A. R., Fanning, K., Netzel, M., Stanley, R., and Singh, I. (2015). Consumption of anthocyanin-rich Queen Garnet plum juice reduces body weight by increasing energy expenditure and plasma glucose level. J. Assoc. Off. Anal. Chem. 15:280. doi: 10.1186/s12870-015-0664-5

Shin, J., Park, E., and Choi, G. (2007). PIFS regulates anthocyanin biosynthesis in an HY5-dependent manner with both factors directly binding anthocyanin biosynthetic gene promoters in Arabidopsis. Plant J. 49, 981–994. doi: 10.1111/j.1365-313X.2006.03021.x

Shukitt-Hale, B., Kalt, W., Carey, A. N., Vinquist-Tymchuk, M., McDonald, J., and Joseph, J. A. (2009). Plum juice, but not dried plum powder, is effective in mitigating cognitive deficits in aged rats. Nutrition 25, 567–573. doi: 10.1016/j.nut.2008.10.018

Song, S., Qi, T., Fan, M., Zhang, X., Gao, H., Huang, H., et al. (2012). The bHLH subgroup IIId factors negatively regulate jasmonate-mediated plant defense and development. PLoS Genet. 9:e1003653. doi: 10.1371/journal.pgen.1003653

Takos, A., Jaffe, F., Jacob, S. B., Robinson, S., and Walker, A. (2006). Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. Plant Physiol. 142, 1216–1232. doi: 10.1104/pp.106

Tamura, Y., Sasaki, N., and Ohmiya, A. (2008). Biosynthesis of plant pigments: anthocyanins, betalains, and carotenoids. Plant J. 54, 733–749. doi: 10.1111/j.1365-313X.2008.03447.x

Usenik, V., Štampar, F., and Veberič, R. (2009). Anthocyanins and fruit colour in plums (Prunus domestica L.) during ripening. Food Chem. 114, 529–534. doi: 10.1016/j.foodchem.2008.09.083

Valero, D., Díaz-Mula, H. M., Zapata, P. J., Guíllen, F., Martínez-Romero, D., Castillo, S., et al. (2013). Effects of alginic edible coating on preserving fruit quality in four plum cultivars during postharvest storage. Postharvest Biol. Technol. 77, 1–6. doi: 10.1016/j.postharvbio.2012.10.011

Vimolmangkang, S., Jeong, C. Y., Kang, G.-H., Yoo, S.-D., Hong, S.-W., and Lee, K. (2015). The Arabidopsis proteins that regulate anthocyanin biosynthesis: quantitative interaction kinetics by Microscale Thermophoresis. Phytochemistry 111, 21–26. doi: 10.1016/j.phytochem.2015.01.004

Wang, L., Feng, Z., Wang, X., Wang, X., and Zhang, X. (2010). DGEseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics 26, 136–138. doi: 10.1093/bioinformatics/btp162

Wu, Z., Meng, D., Wang, A., Li, T., Jiang, S., Cong, P., et al. (2013). The methylation of the MdMYB10 promoter is associated with green-skinned fruit in the ‘Red Barlett’ pear. Plant Physiol. 162, 885–896. doi: 10.1104/pp.113.214700

Wei, H., Chen, X., Zong, X., Shu, H., Gao, D., and Liu, Q. (2015). Comparative transcriptome analysis of genes involved in anthocyanin biosynthesis in the red and yellow fruits of sweet cherry (Prunus avium L.). PLoS ONE 10:e0121164. doi: 10.1371/journal.pone.0121164

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorfull model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126, 485–493. doi: 10.1104/pp.126.2.485

Wrolstad, R. E., Culbertson, J. D., Cornell, C. W., and Mattick, L. R. (1982). Detection of adulteration in blackberry juice concentrates and wines. J. Assoc. Off. Anal. Chem. 65, 1417–1423.

Wu, J., Fu, L., and Yi, H. (2016). Genome-wide identification of the transcription factors involved in citrus fruit ripening from the transcriptomes of a late-ripening sweet orange mutant and its wild type. PLoS ONE 11:e0154330. doi: 10.1371/journal.pone.0154330

Wu, J., Zhao, G., Yang, Y.-N., Le, W.-Q., Khan, M., Zhang, S.-L., et al. (2013). Identification of differentially expressed genes related to coloration in red/green mutant pear (Pyrus communis L.). Tree Genet. Genomes 9, 75–83. doi: 10.1007/s11295-012-0534-3

Wu, Z.-G., Jiang, W., Mantri, N., Bao, X.-Q., Chen, S.-L., and Tao, Z.-M. (2015). Transcriptome analysis reveals flavonoid biosynthesis regulation and simple sequence repeats in yam (Dioscorea alata L.) tubers. BMC Genomics 16:346. doi: 10.1186/s12864-015-1547-8
Xie, X., Li, S., Zhang, R., Zhao, J., Chen, Y., Zhao, Q., et al. (2012). The bHLH transcription factor MdbHLH3 promotes anthocyanin accumulation and fruit colouration in response to low temperature in apples. Plant Cell Environ. 35, 1884–1897. doi: 10.1111/j.1365-3040.2012.02523.x

Xie, Y., Tan, H., Ma, Z., and Huang, J. (2016). DELLA proteins promote anthocyanin biosynthesis via sequestering MYB/L2 and JAZ suppressors of the MYB/bHLH/WD40 complex in Arabidopsis thaliana. Mol. Plant. 9, 711–721. doi: 10.1016/j.molp.2016.01.014

Xu, F., Ning, Y., Zhang, W., Liao, Y., Li, L., Cheng, H., et al. (2013). An R2R3-MYB transcription factor as a negative regulator of the flavonoid biosynthesis pathway in Ginkgo biloba. Funct. Integr. Genomics 14, 177–189. doi: 10.1007/s10142-013-0352-1

Xu, W., Peng, H., Yang, T., Whitaker, B., Huang, L., Sun, J., et al. (2014). Effect of calcium on strawberry fruit flavonoid pathway gene expression and anthocyanin accumulation. Plant Physiol. Biochem. 82, 289–298. doi: 10.1016/j.plaphy.2014.06.015

Xuan, J., Jia, Z., Qain, M., Zhang, J., Wang, G., and Guo, Z. (2015). Comparative of methods for RNA extraction from plum (Prunus salicina Lindl.) fruit flesh. North. Hort. 110–113. doi: 10.1093/jxb/erv524

Ye, J., Hu, T., Yang, C., Li, H., Yang, M., Ijaz, R., et al. (2015). Transcriptome profiling of tomato fruit development reveals transcription factors associated with ascorbic acid, carotenoid and flavonoid biosynthesis. PLoS ONE 10:e0130885. doi: 10.1371/journal.pone.0130885

Copyright © 2016 Fang, Zhou, Ye, Jiang and Pan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.