Transcriptome analysis of colouration-related genes in two white-fleshed nectarine varieties and their yellow-fleshed mutants

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ABSTRACT
Nectarine, a variety of peach that is rich in bioactive and antioxidant compounds, and both white- and yellow-fleshed nectarine fruits are commercially popular. However, although anthocyanin and carotenoid biosynthetic pathways play important roles in plant colouration, the molecular basis of fruit flesh colouration remains largely unknown. Accordingly, we performed an RNA sequencing-based transcriptome analysis of two white-fleshed nectarines, Zhongyou9 (ZY9) and Zhongyou13 (ZY13-W), and their yellow-fleshed mutants, Hongyu (HY) and Zhongyou13-Y (ZY13-Y). To differentiate the impact of ripening on gene expression, we also compared ZY9 and HY at two different time points, namely, before and after fruit ripening. We found that the yellow-fleshed HY may accumulate flavonoids through the generation of more flavonoid biosynthesis precursors, as well as through the overexpression of flavonoid synthase genes. In addition, we also found that HY could impede carotenoid degradation via beta-carotene 3-hydroxylase and carotenoid cleavage dioxygenase genes. Meanwhile, ZY13-Y may regulate its yellow colouration through overexpression of chalcone synthase genes, in order to accumulate flavonoids, as well as through the underexpression of flavonol 3-O-methyltransferase and carotenoid cleavage dioxygenase genes, in order to slow flavonoid and carotenoid degradation. As demonstrated by the multiple transcription factors that were differentially expressed in the yellow- and white-fleshed varieties, it is clear that the regulation of peach and nectarine colouration is a complex process. Furthermore, we also identified several other genes that are potentially related to fruit colouration. Thus, our results provide a valuable advancement in the elucidation of the molecular basis of flesh colouration in nectarine.

Introduction
Nectarine is a variety of peach (Prunus persica [L.] Batsch) that is a model plant in the Rosaceae family and for which a completely sequenced genome is available [1]. Because its fruits are rich in bioactive and antioxidant substances, such as phenolic compounds and vitamin C, nectarine is becoming increasingly popular, and both white- and yellow-fleshed nectarine fruits are favoured by consumers.

Fruit colour is mainly controlled by anthocyanin and carotenoid pathways. Anthocyanin regulation mainly involves transcription-level regulation by transcription factors (TFs) [2]. In this regard, Espley et al. [3] identified multiple repeats in the promoter of MYB10, an R2R3 MYB TF subfamily member that can serve as an autoregulatory locus to increase MYB10 transcript levels and cause anthocyanins to accumulate in red apple fruits [3,4]. Other studies have further associated MYB TFs with red flesh phenotypes in apple, and R2R3 MYB TFs can also regulate the expression of anthocyanin biosynthetic genes, along with co-activators of other TFs, such as the basic-helix-loop-helix (bHLH) and WD40 repeat families [5,6]. Furthermore, Rahim et al. [7] demonstrated that the overexpression of MYB10.1/bHLH3 and MYB10.3/bHLH3 combinations could activate anthocyanin production by upregulating the expression of the anthocyanin biosynthetic genes NtCHS, NtDFR and NtUFGT in peach fruits. Zhou et al. [8] also found that an NAC (NAM, ATAF1/2 and CUC2) domain TF gene, named BLOOD (BL), is responsible for the blood-red flesh trait in peach and that the heterodimer of BL and PpNAC1 can activate the transcription of PpMYB10.1 to produce anthocyanin pigmentation in tobacco. The Peace (peach anthocyanin colour enhancement) gene, which encodes...
a TT2-like R2R3 MYB, has also been shown to regulate anthocyanin biosynthesis and is responsible for pigmented flowers [9]. Glycosylation and methylation, two post-translational modifications, are also involved in the regulation of anthocyanin metabolism. Two flavonoid 3-O-glycosyltransferase (UGT) genes, PpUGT78A1 and PpUGT78A2, and two anthocyanin O-methyltransferase (AOMT) genes, PpAOMT1 and PpAOMT2, have been identified in peach [10].

Meanwhile, the regulation of fruit colouration via carotenoid pathways mainly entails post-transcriptional regulation via carotenoid retention or degradation. For example, Ohmiya et al. [11] determined that the chrysanthemum carotenoid cleavage dioxygenase gene (CmCCD4a) is responsible for the white petal phenotype observed in the species, since CmCCD4a is associated with the degradation of carotenoids into colourless apocarotenoids. Similarly, in peach, PpCCD4 is widely reported to control carotenoid degradation. Brandi et al. [12] revealed that CCDs play an important role in determining the flesh colour phenotypes and volatile composition of ‘Redhaven’ peach and its white-fleshed mutant. In addition, Falchi et al. [13] systematically studied PpCCD4 genotypes in 37 peach varieties with different flesh colour phenotypes and found that three independent mutational events – nucleotide substitutions, small insertions, and transposable element insertions – have taken place in the PpCCD4 gene and can alter its expression. Ma et al. [14] performed linkage mapping with peach F1 populations, in order to narrow the peach yellow-flesh locus to a 2.6-cM interval, and using RNA sequencing (RNA-seq) and quantitative real-time PCR (qRT-PCR) analysis, they also determined that CCD4 transcript levels were consistent with carotenoid degradation in peach fruits. These pieces of evidence suggest that PpCCD4 is responsible for the white and yellow colouration of peach fruit flesh. Fukamatsu et al. [15] also observed mutations in CCD4 in yellow-fleshed peaches. The metabolism of phenolic compounds may also play an important role in fruit flesh colouration. In particular, Gabotti et al. [16] revealed that cinnamyl alcohol dehydrogenase genes contribute to the texture of blood red-fleshed fruit.

RNA-seq, a widely applied transcriptome profiling method, has been used to study the function of peach colouration-related genes [17]. For example, Ma et al. [14] used RNA-seq, along with linkage mapping, to reveal that PpCCD4 is responsible for the yellow colouration in peach fruit flesh and leaf midveins. Drawing on linkage mapping information from previous studies, Zhou et al. [18] performed a transcriptome comparison between red and green peach leaves and identified PpMYB10.4, which was located in the Gr interval on LG6, as the gene responsible for anthocyanin accumulation and red colouration. In a high-throughput sequencing (Roche 454 GS FLX)-based transcriptome analysis, Chen et al. [19] also identified 514 differentially expressed genes (DEGs) between red and white flower petals from a variegated peach tree. Therefore, we used RNA-seq to study the genetic basis of fruit flesh colour in two white-fleshed nectarines, Zhongyou9 (ZY9) and Zhongyou13 (ZY13-W), and their yellow-fleshed mutants, Hongyu (HY) and Zhongyou13-Y (ZY13-Y). To differentiate the impact of ripening on related gene expression, we also compared ZY9 and HY harvested at two different time points (before and after ripening, designated as ZY9-1/HY-1 and ZY9-2/HY-2, respectively). RNA-seq was performed to profile the nectarine fruit flesh transcriptome and to identify DEGs between the white- and yellow-fleshed pairs (i.e. between ZY9 and HY and between ZY13-W and ZY13-Y). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses revealed multiple colouration-related, anthocyanin (flavonoid), and carotenoid biosynthetic pathway genes, as well as TF genes, some of which were novel. Our results should be beneficial for the elucidation of the molecular basis of fruit flesh colour in nectarine.

Materials and methods
Sample preparation and total RNA extraction

Fresh fruit, which mature at the 70th day after anthesis, were obtained from nectarine varieties HY and ZY9 on June 17, 2014 (prior to ripening) and July 9, 2014 (at ripening) from the experimental station of the Department of Horticulture, Anhui Academy of Agricultural Sciences, China. Fruit flesh of ZY13-Y and ZY13-W was only obtained at the ripening stage; collection was done in Dangshan, Anhui. Flesh from several fruits from the same nectarine tree was pooled together and subjected to total RNA extraction using an Invitrogen Trizol kit (Ambion, Texas, United States). RNA yield was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA) and RNA integrity was evaluated using agarose gel electrophoresis with ethidium bromide staining.

Library construction and sequencing

After obtaining high-quality RNA, cDNA was synthesized using random hexamer primers. We selected 200–500-bp fragments for PCR amplification to generate the sequencing libraries, and an Agilent 2100 Bioanalyzer and an ABI Step One Plus Real-Time PCR system were used to quantify and qualify the constructed libraries,
respectively. Libraries of suitable quality were sequenced on an Illumina HiSeq 2000 system in the form of 90-bp paired-end reads. The original image data were transformed into sequence data via base calling. The raw reads which were defined as ‘clean’ reads through quality-check were used in the subsequent bioinformatics analysis.

Bioinformatics analysis
We first used SOAPaligner/SOAP2 to align the clean reads to the peach reference genome and transcripts [1,20,21]. The alignment was then quality-checked with SOAPaligner/SOAP. To identify DEGs, relative gene expressions were calculated using the FPKM method and compared among samples [22]. DEGs between pairs of samples were detected using the criteria of FDR ≤ 0.001 and |Log2Ratio| ≥ 1 [23]. To further evaluate DEG functions, Cluster [24] and Java Treeview [25] software packages were used. KEGG [26] pathway enrichment analyses were performed to discover enriched pathways. To refine gene structure, we used Cufflink [27] for transcript assembly to find new transcripts and to extend the 5’ and 3’ gene ends. Coding Potential Calculator [28] was used to assess the protein-coding potential; and to identify alternative splicing events, we used TopHat [29] because of its outstanding performance. Finally, SOAPsnp [20] and SOAPfuse [30] were used to detect single nucleotide polymorphisms and fused genes, respectively.

qRT-PCR
The quantification of gene expression was performed using a two-step reaction process: reverse transcription (RT) and PCR amplification. The RT reactions were performed in a GeneAmp PCR System 9700 (ABI) using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). Meanwhile, real-time PCR was performed on a LightCycler 480 II Real-time PCR Instrument (Roche, Basel, Switzerland) using SYBR® Premix Ex Taq™ (Tli RNAseH Plus). The RT-PCR reactions were performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Primer sequences (Supplementary Table S1), which were designed in the laboratory and synthesized by Generay Biotech (Shenzhen, China), were based on mRNA sequences obtained from the NCBI database. The mRNA expression levels were normalized to 18S rRNA and were calculated using the 2−ΔΔCt method [31].

Results and discussion
RNA-seq and performance
RNA-seq yielded an average of 48 Mb of 92-bp clean reads (i.e. 4.3 Gb of clean data; Table 1) and a total of 1881, 1959 and 1270 DEGs were identified between HY-1 and ZY9-1, HY-2 and ZY9-2, and ZY13-Y and ZY13-W, respectively (Supplementary Table S2).

To test the RNA-seq performance, five upregulated genes and five downregulated genes were selected (the primers are listed in Supplementary Table S1). We found that 70% (21 of 30) of the qRT-PCR results were consistent with those from RNA-seq (Figure 1), and the observed inconsistencies between the qRT-PCR and RNA-seq results may have occurred as a result of the very low transcript levels of the randomly selected genes.

Differentially expressed genes
Anthocyanin (flavonoid) and carotenoid biosynthetic pathways play important roles in fruit colouration. We identified 72 putative flavonoid and carotenoid biosynthesis genes and 8 TF-encoding genes that could potentially regulate flavonoid biosynthetic genes. Together, we considered these to be ‘known’ colouration-related genes. Thus, all identified peach TFs, MADS-box TFs and microRNA-targeted TFs were treated as ‘candidate’ colour-related genes (Supplementary Table S3) in that TFs play important roles in the control of fruit colour development. All known and candidate genes identified as DEGs were also annotated and compiled (Supplementary Table S4). We identified 419 upregulated and 102 downregulated DEGs (Supplementary Table S5) in the HY/ ZY9 pair and we also identified 377 upregulated and 893 downregulated DEGs in the ZY13-Y/ZY13-W pair (Supplementary Table S6). We constructed a Venn diagram of the HY-1/

Table 1. RNA-seq performance.

| Sample name | Clean reads (90 bp) | Clean bases | Genome map rate | Expressed gene | Novel transcripts | Alternative splicing | SNP |
|-------------|---------------------|-------------|-----------------|---------------|------------------|---------------------|-----|
| HY-1        | 48,290,784          | 4,346,170,560 | 84.40%          | 19,323        | 893              | 18,718              | 19,628 |
| HY-2        | 48,191,506          | 4,337,235,540 | 83.87%          | 19,339        | 808              | 18,678              | 19,366 |
| ZY13-W      | 48,163,250          | 4,334,692,500 | 85.19%          | 18,859        | 765              | 17,562              | 19,051 |
| ZY13-Y      | 48,240,156          | 4,314,759,300 | 86.25%          | 18,724        | 759              | 18,412              | 18,693 |
| ZY9-1       | 48,240,980          | 4,342,408,200 | 85.14%          | 18,945        | 757              | 17,426              | 18,793 |
| ZY9-2       | 48,240,156          | 4,341,614,040 | 84.63%          | 19,278        | 845              | 18,782              | 19,903 |

SNP: single nucleotide polymorphism

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ZY9-1, HY-2/ ZY9-2, and ZY13-Y/ ZY13-W DEGs (Figure 2). In addition, 110 DEGs were shared by HY-2/ ZY9-2 and ZY13-Y/ ZY13-W (Supplementary Table S7).

**Flavonoid and carotenoid biosynthetic pathway-related genes**

Flavonoid and carotenoid biosynthetic pathway genes are widely known to control the colouration of fruit skin and flesh. For instance, CCD, a protein encoded by one of these genes, is involved in the formation of white colouration that is associated with carotenoid degradation which contributes to the white-fleshed peach phenotype [12,13,15]. In our data, we found that flavonoid and carotenoid biosynthetic pathway genes were enriched (Figure 3) and identified (Figures 4 and 5).

In the HY/ ZY9 pair, ppa006109m, which is a putative CCD4 gene, was significantly downregulated in all three

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**Figure 1.** Comparison of RT-PCR and RNA-seq results. HY-1 and ZY9-1 (A), HY-2 and ZY9-2 (B), ZY13-Y and ZY13-W (C). Note: Y-axes show log2Ratio of QPCR or FPKM value. Red indicates expression from RNA-seq's Log2Ratio of FPKM, and blue indicates expression from RT-PCR.
Figure 2. Venn diagram of differentially expressed genes common to the three wild-type/mutant pairs of nectarine varieties.

yellow-fleshed libraries and, thus, may be responsible for carotenoid accumulation and the development of yellow colour in HY and ZY13-Y. In addition to ppa006109m, we also detected two other CCD genes, ppa002804m and ppa002314m. We observed that ppa006000m, a putative prephenate dehydratase (PDT) gene, was significantly upregulated in the HY-2 library, which could potentially lead to the accumulation of phenylalanine, a precursor for flavonoid biosynthesis. Meanwhile, ppa025745m, a putative chalcone synthase (CHS) gene, was also upregulated in HY-2 and could also contribute to flavonoid accumulation. In contrast, ppa009001m, a putative beta-carotene 3-hydroxylase gene, was significantly downregulated in HY-2, which could impede the degradation of beta-carotene to zeaxanthin. Therefore, these DEGs might contribute to the yellow flesh colour of HY-2, by generating more precursors for flavonoid biosynthesis, overexpressing flavonoid synthase genes and impeding carotenoid degradation.

Meanwhile, in the ZY13-W/ZY13-Y pair, we found that three putative flavonoid synthase genes (ppa006899m, ppa008402m, and ppa023080m) were significantly upregulated in yellow-fleshed ZY13-Y, which could cause flavonoid accumulation. In contrast, the CCD gene ppa006109m and the putative flavonol 3-O-methyltransferase (3-OMT) gene ppa007511m were significantly downregulated, which could impede alysin biosynthesis and flavonoid degradation, thus contributing to the yellow-flesh phenotype of ZY13-Y. This result is consistent with the findings of Schmidt et al. [32], who reported that flavonol 3-OMT contributes to the accumulation of highly methylated colourless myricetin compounds in the trichomes of the wild tomato species Solanum habrochaites. Therefore, the yellow-fleshed nectarine ZY13-Y may regulate its colouration in two ways: 1) by overexpressing CHS genes (to accumulate flavonoids) and 2) by lowering the expression of flavonol 3-OMT and CCD genes (to retard flavonoid and carotenoid degradation). In addition to these genes related to the colouration of peach flesh, we also identified several novel colouration-related genes worthy of further investigation (Table 2).

Transcription factors and nectarine colouration

The anthocyanin biosynthetic pathway has been widely studied and found to be associated with TF regulation, including MYB families and other TF families, such as bZIP, NAC and bHLH. By systematically searching peach TFs and evaluating differentially expressed TF genes in the white-/yellow-fleshed nectarine pairs, we identified many shared TFs between HY-2/ZY9 and ZY13-Y/ZY13-W (Table 3). Ten TF genes (ppa001495m, ppa008801m, ppa015954m, ppa011221m, ppa011644m, ppa026704m, ppa006561m, ppa004359m, ppa007593m and ppa006134m) were downregulated and two ones (ppa024027m and ppa021277m) were upregulated in yellow-fleshed HY and ZY13-Y. Among the above-mentioned TFs, the R2R3 MYB TF family has been widely studied. For example, Ravaglia et al. [33] demonstrated that MYB10 can positively regulate the expression of the anthocyanin biosynthetic genes UFGT and dihydroflavonol 4-reductase in peach, and Zhou et al. [18] further associated PpMYB10.4 with anthocyanin accumulation and red leaves. In the present study, we observed that ppa015954m, a putative MYB family TF gene, was downregulated. Interestingly, other TF families, such as bHLH, basic leucine zipper (bZIP), NAM, ATAF, CUC (NAC), have been reported as differentially expressed in the pulp of a red mutant of orange [34]. Nevertheless, the associated regulatory mechanism remains largely unknown. Akagi et al. [23] reported that the overexpression of the bZIP TF gene DkbZIP5 in persimmon (Diospyros kaki) calluses upregulated the expression of the MYB TF gene DkMyb4 and resulted in the accumulation of proanthocyanidins. In the present study, we found that ppa007593m, a putative bZIP family TF gene, was downregulated in the yellow-fleshed nectarines. Zhou et al. [8] demonstrated that PpNAC1, an NAC family TF gene, can activate PpMYB10.1 and regulate anthocyanin biosynthesis in blood red-fleshed peach. In the present study, we found that ppa008801m, a putative NAC family TF gene, was downregulated in the yellow-fleshed nectarines. Finally, Espley et al. [35] reported that MdMYB10 can regulate anthocyanin biosynthesis and contribute to red colouration in apple and that such regulation was dependent on the
co-expression of two distinct bHLH genes, MdbHLH3 and MdbHLH33. We observed that ppa006134m, a putative hHLH family TF gene, was downregulated in the yellow-fleshed nectarines. The interaction between MYB and bHLH TFs can regulate the expression of another TFs. Ishida et al. [36] found that R2R3 MYB and bHLH TFs can form a complex to regulate WRKY TF expression. In the present study, two WRKY family TF genes, ppa026704m and ppa024027m, were down- and upregulated, respectively, in the yellow-fleshed nectarines. To understand the function of TF genes in fruit flesh colouration, additional functional studies are needed.
Figure 4. Anthocyanin/flavonoid biosynthetic pathway-related genes differentially expressed in white-/yellow-fleshed nectarine pairs.

Figure 5. Carotenoid biosynthetic pathway-related genes differentially expressed in white-/yellow-fleshed nectarine pairs.
Differentially expressed fruit coloured nectar colouration-related genes common to HY/ ZY9 and ZY13-Y/ ZY13-W.

| Gene ID   | Regulation model | Annotation                                                                 |
|-----------|------------------|-----------------------------------------------------------------------------|
| ppa025208m | up               | Actin-binding FH2/DRF autoregulatory; GO:0003779 actin binding;              |
| ppa024163m | up               | Xb;                                                                         |
| ppa003428m | up               | Pyruvate/Phosphoenolpyruvate kinase; GO:0003824 catalytic activity; E4.1.3.1, aceA; isocitrate lyase [EC:4.1.3.1] |
| ppa012956m | up               | Protein of unknown function                                                |
| ppa006109m | down             | Carotenoid oxygenase; carotenoid genes, Nced; 9-cis-epoxycarotenoid dioxygenase [EC:1.13.11.51] |
| ppa005405m | down             | EGF-like, allinase; Pyridoxal phosphate-dependent transferase, major region, subdomain 2; GO:0003824 catalytic activity; |
| ppa012123m | down             | Ribulose bisphosphate carboxylase, small chain; ribC5; ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39] |
| ppa009844m | down             | Glycoside hydrolase, family 19, catalytic; GO:0004568 chitinase activity; E3.2.1.14; chitinase [EC:3.2.1.14] |
| ppb022507m | down             | Auxin responsive SAUR protein;                                              |
| ppa011739m | down             | Chlorophyll a/b-binding protein domain;                                     |
| ppa009537m | down             | Cupin, RmlC-type; GO:0005114 oxidation-reduction process;                   |
| ppa013990m | down             | Protein of unknown function wound-induced;                                 |

Table 3. Transcription factors common to HY/ ZY9 and ZY13-Y/ ZY13-W.

| Gene ID   | Regulation model | Annotated transcription factors                                                                 |
|-----------|------------------|-----------------------------------------------------------------------------------------------|
| ppa01495m | down             | putative transcription factors: TALE family                                                  |
| ppa00801m | down             | putative transcription factors: NAC family                                                   |
| ppa01595m | down             | putative transcription factors: MYB family                                                   |
| ppa01121m | down             | putative transcription factors: HD-ZIP family                                                |
| ppa01164m | down             | putative transcription factors: bZIP family                                                  |
| ppa02670m | down             | putative transcription factors: WRKY family                                                  |
| ppa006561m| down             | putative transcription factors: G2-like family                                               |
| ppa004359m| down             | putative transcription factors: C2H2 family                                                  |
| ppa007593m| down             | putative transcription factors: bZIP family                                                  |
| ppa006134m| down             | putative transcription factors: bHLH family                                                  |
| ppa024027m| up               | putative transcription factors: WRKY family                                                 |
| ppa021277m| up               | putative transcription factors: GRF family                                                  |

Conclusions

In summary, the gene expression differences between the wild-type white- and mutant yellow-fleshed nectarines were far more complex than previously reported. Although only a few DEGs overlapped between the two wild-type/mutant pairs, many were putative flavonoid and carotenoid synthase or degradation-related genes, and, many novel flavonoid and carotenoid pathway-related genes and novel TF genes were newly identified, but, require further evaluation. Our findings should help other researchers elucidate the molecular basis of fruit flesh colouration.

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Disclosure statement

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