

Transcriptomic and Metabolomic Analysis of Anthocyanin and Carotenoid Biosynthesis in Red and Yellow Fruits of Sweet Cherry (*Prunus avium* L.) during Ripening

Qinghao Wang

Zhejiang University

Luyang Jing

Zhejiang University

Yue Xu

Zhejiang A&F University

Weiwei Zheng

Zhejiang A&F University

Wangshu Zhang (✉ zjuzws@zju.edu.cn)

Zhejiang University

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Abstract

Diversity of fruit color in sweet cherry (*Prunus avium* L.) has been attributed to the presence of either anthocyanin or carotenoid. We profiled the anthocyanin and carotenoid metabolites to investigate the most different pigments and the underlying regulatory mechanisms of differential expression genes (DEGs) between red and yellow fruits of sweet cherry.

Profiled the two cultivars, 'Jiangnanhong' (JNH, red fruits) and 'Chaoyang' (CY, yellow fruits) anthocyanin and carotenoid metabolites by LC-MS/MS and transcriptome analysis by RNA-seq to test the difference of gene expression and metabolic substances between the two varieties.

Cyanidin-3-*O*-rutinoside was the most different pigment between two cultivars, the content of which in red fruit was significantly higher than yellow one during the whole ripening stage (stage 3 and stage 4). Total carotenoid content in the two color types of fruits were close, but the content in yellow fruit showed more stable after harvest. Based on the transcriptome data, the heatmap of selected structural DEGs showed that all of the anthocyanin genes expressed significantly higher levels in red fruits than that in yellow fruits. Two unigenes encoding chalcone synthase (CHS) and UDP glucose-flavonoid 3-*O*-glucosyltransferase (UGFT) expressed 1134.58 and 1151.24 times higher in red than in yellow fruits at stage 4, respectively.

Correlation analysis showed that anthocyanin genes in JNH were negatively correlated with that in CY, contrastly, there were some strong correlations were observed between two cultivars in carotenoid genes. Thus, the coloration of sweet cherry was mainly attributed to anthocyanin-related genes.

1 Introduction

Sweet cherry (*Prunus avium* L.), with attractive appearance, desirable taste, dietary properties and various biological activities, which not only directly influence consumer choice, but also are important directions of new fruit varieties selection and breeding today (Nawirska-Olszańska et al., 2017). The color of the sweet cherry fruit ranges from dark red to yellow is determined by the pigments present (e.g. anthocyanins, carotenoids, etc.). Most sweet cherry species in red color are particularly rich in anthocyanins, and are predominantly cyanidin glycosides (Gonçalves et al., 2007; González-Gómez et al., 2010; Liu et al., 2013).

Anthocyanin is a class of water-soluble natural pigment in plants that widely presents in the nature belongs to a subgroup of flavonoids. To date, there have been more than 635 anthocyanins identified, and approximately 95% of which are derived from 6 anthocyanins: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin (He and Giusti, 2010; Eder, 2000; Kong et al., 2003). Various enzymes have been reported to be involved in the anthocyanin biosynthesis process, including chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), dihydroflavonol-reductase (DFR), and anthocyanidin synthase (ANS) (Koes and Verweij, 2005). The mechanisms of anthocyanin biosynthesis have been elucidated in a variety of plant species, such as apple, peach, sweet cherry, eggplant, tomato, etc (Meng et al., 2015; Ye et al., 2019; Wei et al., 2015; Li et al., 2021; Sun et al., 2019). *UGFT* family showed differences between red and non-red apple fruits in previous study. Among them, *UGFT2* was up-regulated only in non-red cultivar, while *UGFT4* was up-regulated only in red cultivar, indicating that *UGFT* play an important role in anthocyanin accumulation in apple skin of different colors (Meng et al., 2015). In the epidermis of purple eggplant (*Solanum melongena*), MYB86 could directly bind to the promoters of *CHS*, *F3H*, and *ANS* and suppress their activities, resulting to the reduction of the anthocyanin (Li et al., 2021). In sweet cherry, the anthocyanin content increased gradually during the development, especially from the young fruits to the breaker fruits stage. Some unigenes have been reported to be up-regulated in red-colored fruit, including *4CL*, *CHS*, *CHI*, *F3H*, *DFR*, etc.

Among them, *UFGT* was suggested to play a key role in red and bicolored (red and yellow) fruits, and the expression of which are maintained at high levels in red cultivars in the late ripening stage. Some transcription factor families, such as MYB, bHLH and WD40, may involved in anthocyanin biosynthesis control (Wei et al., 2015; Liu et al., 2013).

Carotenoids are a class of widespread secondary metabolites that are crucial to humans and plants, mainly divided into α -carotene, β -carotene, lycopene, lutein, astaxanthin and zeaxanthin. Carotenoids are not synthesized by humans but are essential for several functions, so they must be obtained through the diet, especially fresh fruits (Luo et al., 2021; Kopec and Failla, 2017). To date, many data have been available related to the molecular characterization of genes involved in carotenoid biosynthesis. Hu et al. (2021) elucidated that one *LCYE* gene (LOC113688784) expressed 2.21 times higher in matured seeds of red coffee (*Coffea arabica*) than that in yellow coffee seeds. Li et al. (2021) found that the imbalanced regulation of *HYE* and *HYB* resulted in an increase of zeaxanthin and a decrease of lutein in Chinese raspberry (*Rubus chingii*). Likewise, Wu et al. (2020) reported that down-regulation of transcription factor *SIMYB72* of R2R3-MYB promoted β -carotene production and decreased lycopene content in tomato (*Solanum lycopersicum*). Despite these findings, the molecular regulation mechanism of carotenoid biosynthesis between different color sweet cherry fruits remained unclear.

RNA-seq analysis is commonly used to identify the differential genes and understand their mechanism underlying gene expression regulation in the biosynthetic pathway of different species from a transcriptomic perspective. Based on the transcriptome analysis, scientists have found that the reduction of key anthocyanin gene expression at the late stage of in white bayberry (*Morella rubra*) compared with red bayberry fruits, resulting in the failure to turning red (Lin, Zhong and Zhang, 2019). Wei et al.(2015) obtained the transcriptome and DGE profiling data to compare the transcripts involved in the anthocyanin biosynthesis. However, for carotenoid biosynthesis, reports on the transcriptional regulation at specific ripening stages and finally determines the quality in red and yellow sweet cherry are still limited. Herein, in order to identify the major disparity of pigmental substance and the molecular mechanisms in anthocyanin and carotenoid biosynthesis of sweet cherry, we have explored the anthocyanin and carotenoid compounds and transcriptome profiling of a red cultivar (JNH) and a yellow cultivar (CY) in four ripening stages.

2 Materials And Methods

2.1 Samples

Two cultivars, 'Jiangnanhong' (JNH, red fruits) and 'Chaoyang' (CY, yellow fruits) used for this study have been successfully cultivated in some regions of southeast China (Fig. 1). All the samples of JNH and CY were collected from a orchard in Yuyao county, Zhejiang province, China (121°16' E, 30°1' N; elevation 6m). Three individual trees were selected for each cultivar, and the fruits were harvested from each tree at 4 ripening stages: 20 days after flowering (20 DAF, stage 1), 27 DAF (stage 2), 34 DAF (stage 3), 41 DAF (stage 4) (Fig. 2A). Additionally, in order to further explore the changes of pigment contents after harvesting, the fruits of JNH and CY were still collected every 2 days until the color no longer changed, 5 and 3 maturity levels of two cultivars were obtained (Fig. 2B). All samples were immediately placed in ice box and taken back to the laboratory, blended and ground into powder in liquid nitrogen, the RNA-seq samples (JNH and CY fruits in 4 ripening stages) were encapsulated in dry ice and sent to BGI Genomics Co., Ltd (Wuhan, China) for transcriptome sequencing, the remaining samples were preserved at - 80 °C for subsequent research.

2.2 Determination of anthocyanin and carotenoid content

Anthocyanin content was determined using the method described by Wei et al. (2015) with minor modification. The anthocyanins were extracted in 10 mL methanol (containing 0.05% hydrochloric acid) for 24h at 4°C in darkness. The anthocyanin content was measured by the TripleTOF 5600 LC-MS/MS (AB SCIEX, USA) at 520 nm. Anthocyanin compound standards, including cyanidin-3-*O*-glucoside (C3G), cyanidin-3-*O*-rutoside chloride (C3R) and pelargonidin-3-glucoside chloride (P3G) were purchased from Sigma Aldrich Chemical Co. (Mo., USA).

Carotenoid content was determined in three replications. 1g of the sample powder (pre-ground in liquid nitrogen) was homogenized with an extraction solution (5ml, 80% pre-cooled acetone) and incubated overnight at 4°C in the dark. Next, the samples were centrifuged at 7500 rpm for 20 min at 4 °C and absorbance was measured spectrometrically at 470nm (80% acetone were used as a control group). Carotenoid content was calculated according to Dere, Günes and Sivaci (1998), and expressed as µg/g fresh weight.

2.3 Total RNA extraction

Total RNA was extracted by the polysaccharide polyphenol reagent kit (Takara Cat. 9769, China). The integrity of RNA was determined by 1.5% agarose gel electrophoresis (Fig. 3). The purity and concentration of RNA were determined by Nandrop 2000C (Thermo, USA).

2.4 Library construction and transcriptome sequencing

Oligo (dT)-attached magnetic beads were used to purified mRNA, which was fragmented into small pieces with fragment buffer at appropriate temperature. Then first-strand cDNA was generated using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis. Afterwards, A-tailing mix and RNA index adapters were added by incubating to end repair. The cDNA fragments obtained from previous step were amplified by PCR, and products were purified by Ampure XP beads, then dissolved in EB solution. The product was validated on the Agilent Technologies 2100 bioanalyzer for quality control. The double stranded PCR products were heated denatured and circularized by the splint oligo sequence to get the final library. The single strand circle DNA (ssCir DNA) was formatted as the final library. The final library was amplified with Phi29 polymerase to make DNA nanoball (DNB), which had more than 300 copies of one molecule. DNBs were loaded into the patterned nanoarray and pair end 100 bases reads were generated on BGISEQ500 platform (BGI, China). This project sequencing used the DNBSEQ platform and referenced existing genome (https://www.ncbi.nlm.nih.gov/nuccore/NW_018922178.1) from NCBI. The sequencing raw data are deposited in the NCBI Sequence Read Archive under BioProject ID (Accession No. PRJNA905870).

2.5 Sequencing data filtration and transcripts mapping

The sequencing data was filtered with SOAPnuke (v1.5.2) by (1) Removing reads containing sequencing adapter; (2) Removing reads whose low-quality base ratio (base quality less than or equal to 5) is more than 20%; (3) Removing reads whose unknown base ('N' base) ratio is more than 5% (Li, Kristiansen and Wang, 2008). Afterwards, clean reads were obtained and stored in FASTQ format and then mapped to the reference genome (https://www.ncbi.nlm.nih.gov/nuccore/NW_018922178.1) using HISAT2 (v2.0.4) (Kim, Langmead and Salzberg, 2015). BOWTIE (v2.2.5) was applied to align the clean reads to the gene set, followed by de novo transcript prediction and differentially spliced gene detection (Li and Dewey, 2011).

2.6 Functional gene annotation

The ORF of unigene was detected by GETORF (EMBOSS 6.5.7) package, and the ORF was mapped to the structural domain of transcription factor protein using HMMSEARCH 3.0 package. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database categories were assigned to the unigene sequences using the KEGG Automatic Annotation

Server (KAAS) online. Blast2GO v2.5 was used to obtain Gene Ontology (GO) annotation of unigenes. The gene expression levels were calculated by RSEM (v1.2.12).

2.7 Different expression gene (DEG) analysis

Differential expression analysis was performed using the DESeq R (v1.4.5) package. To take insight to the change of phenotype, the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analysis of annotated different expression gene were performed by Phyper function based on Hypergeometric test (https://en.wikipedia.org/wiki/Hypergeometric_distribution). The significant levels of terms and pathways were corrected by Q-value with a rigorous threshold ($Q \text{ value} \leq 0.05$) by Bonferroni method.

2.8 Quantitative real-time reverse transcription PCR (qRT-PCR) analysis

In order to verify the expression pattern results determined by RNA-seq, six and four unigenes involved in anthocyanin and carotenoid metabolic ways were chosen for qRT-PCR analysis, respectively. Total RNA was extracted by the same method mentioned above. Specific primer pairs and reference gene (*β -Actin*) were showed in S4 table. cDNA was obtained using the ABScript II RT Mix reverse transcription kit (ABclonal co., Ltd, China). qRT-PCR analysis was also performed by a kit according to protocol (Vazyme biotech co., Ltd, China). The following reactions were performed using a real-time PCR instrument (ABI Q6 Flex) with a procedure: 95°C, 1 min; 95°C 15 s; 60°C 1 min (45 cycles of the above steps). Each plate was repeated three times in independent runs for all reference and selected genes. The real-time data were analyzed by CFX Manager™ 3.0 software to obtain amplification and melting curves, which were transformed to analyze the data results by the $2^{-\Delta\Delta CT}$ method. Three biological replicates and three technical replicates were performed for each sample.

2.9 Statistical analysis

The content of each chemical compound was showed as the mean $\pm$ standard deviation of three replicates. Statistical analysis was performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Significant differences were calculated using one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test at the 5% level ($p < 0.05$) and are shown in the tables with lowercase letters (a, b, c, etc.) between samples. Undetectable substances are marked with "nd" in all chemical profile tables. Correlation analysis was carried out by Pearson's test ($p < 0.05$), and correlation figure was drawn with Origin 8.0.

3 Results

3.1. Anthocyanin and carotenoid content in different ripening stages and maturity levels of sweet cherry

Three types of anthocyanins, cyanidin-3-*O*-glucoside (C3G), cyanidin-3-*O*-rutinoside chloride (C3R) and pelargonidin-3-glucoside chloride (P3G), and total carotenoid were identified (Table 3). Among them, C3R was the most abundant anthocyanin in both fruits of JNH and CY, but P3G was almost undetectable. Notably, C3R significantly increased after S3 in JNH, and continued to increase rapidly until postharvest. But in CY, C3R content was always kept at a low level. The content of C3R reached a maximum of 3909.32 $\mu\text{g/g}$ at JNH, but only reached a maximum of 6.25 $\mu\text{g/g}$ in CY, which was only 0.16% of that in JNH. Overall, the anthocyanin content present a prominently higher level in red cherries (JNH) than that in yellow cherries (CY) during the ripening stages and after harvest in this study.

Table 1
The cultivars used in the present study

Cultivars	Scientific name	Abbreviation	Ripening stage ^a	Maturity level ^b
'Jiangnanhong'	<i>Prunus avium</i>	JNH	S1, S2, S3, S4	, , , ,
'Chaoyang'	<i>P. avium</i>	CY	S1, S2, S3, S4	, ,
^a S1, S2, S3, S4 refers to different ripening stages, see Fig. 2A for detail.				
^b , , , , refers to different maturity level, see Fig. 2B for detail.				

Table 2
The morphological trait and quality index value of the cultivars' fruit in fully mature period

Cultivars	Color ^a	a/b ^b	Weight (g)	Length (cm)	Width (cm)	TSS (°Brix) ^c	TA (%) ^d
JNH	Red	2.73 ± 0.43a	8.73 ± 0.69a	21.29 ± 0.88a	25.31 ± 1.25a	15.77 ± 0.49a	0.40 ± 0.02a
CY	Yellow	0.31 ± 0.01b	7.93 ± 0.46a	21.56 ± 0.29a	26.57 ± 0.37a	14.93 ± 1.06a	0.43 ± 0.01a
^a see Fig. 1 for detail.							
^b The ratio of red-green to yellow-blue.							
^c TSS, total soluble solid content.							
^d TA, titratable acidity.							

Table 3

Anthocyanin and carotenoid content in the fruits of cultivars in different developmental stages^a

	Sample	Cyanidin-3- <i>O</i> -Rutinoside(ug/g·FW)	Cyanidin-3- <i>O</i> -Glucoside(ug/g·FW)	Pelargonidin 3-glucoside(ug/g·FW)	Total carotenoid(ug/g·FW)
Ripening stages	JNH S1	0.23 ± 0.01d	4.35 ± 0.35a	nd ^b	2.03 ± 0.05f
	JNH S2	2.62 ± 0.18c	1.46 ± 0.06c	nd	4.22 ± 0.34e
	JNH S3	38.04 ± 2.31b	0.99 ± 0.21d	nd	11.78 ± 0.69b
	JNH S4	719.31 ± 19.84a	1.93 ± 0.06b	0.13 ± 0.01a	11.17 ± 0.86c
	CY S1	nd	0.19 ± 0.03e	nd	2.48 ± 0.14f
	CY S2	nd	0.17 ± 0.07e	nd	7.05 ± 0.07d
	CY S3	0.32 ± 0.01e	0.12 ± 0.02e	nd	11.79 ± 0.28b
	CY S4	2.34 ± 0.21c	0.09 ± 0.02e	nd	12.86 ± 0.38a
Maturity level	JNH	112.62 ± 5.59d	1.00 ± 0.06d	nd	10.63 ± 0.02d
	JNH	111.07 ± 5.17d	1.13 ± 0.04d	nd	9.01 ± 0.10c
	JNH	837.70 ± 15.12c	1.71 ± 0.02c	nd	8.27 ± 0.07b
	JNH	1424.16 ± 7.56b	4.29 ± 0.05b	nd	6.53 ± 0.09e
	JNH	3909.32 ± 112.67a	13.54 ± 0.28a	0.23 ± 0.01a	3.19 ± 0.21f
	CY	1.35 ± 0.23e	0.94 ± 0.03de	nd	13.49 ± 0.06a
	CY	1.44 ± 0.12e	1.07 ± 0.16d	nd	13.75 ± 0.08a
	CY	6.25 ± 0.44e	0.74 ± 0.11e	nd	13.80 ± 0.13a
^a Data expressed as means ± standard deviation of samples' fresh weight in different ripening stages and maturity levels					
^b nd, the substance was not detectable					

With regard to the total carotenoid, its content was much less than the anthocyanin in two cultivars. The amount of total carotenoid content increased gradually during ripening in JNH and CY. However, after maturity, the carotenoid content in CY (13.80 ug/g) was 4.33 times higher than that in JNH (3.19 ug/g). Moreover, an obviously downward trend was observed in the carotenoid content of JNH from maturity level to , whereas the content change in CY appeared more stable (Table 3).

3.2. Transcriptome profile of the RNA libraries of sweet cherry

We constructed eight cDNA libraries from total RNA of JNH_S1, JNH_S2, JNH_S3, JNH_S4, CY_S1, CY_S2, CY_S3, CY_S4. These cDNA libraries were subjected to pair-end reading with BGISEQ500 platform (BGI, Beijing). This RNA-Seq project referenced existing genome (https://www.ncbi.nlm.nih.gov/nuccore/NW_018922178.1) from NCBI (S1 Table).

The preprocessing result of sequencing data was showed in Table 4. It can be seen that after the quality pretreatment of the original sequencing sequence, the minimum percentage of clean reads in raw reads was 93.20%, the minimum of Q30 was 90.94%, and the minimum mapping rate was 86.74%. The assembly statistics were showed in S2 Table. The sequencing and assembly results suggest that the unigene data were highly reliable for further analysis.

Table 4
The quality assessment of sequencing data of cultivars with different packaging materials

Sample	Clean bases (Gb)	Clean reads /Raw reads (%)	Q20 (%)	Q30 (%)	Total mapping (%)
JNH S1	6.38	97.03%	97.44	92.21	86.76
JNH S2	6.44	97.95%	97.32	91.79	88.95
JNH S3	6.43	97.79%	97.05	90.94	88.34
JNH S4	6.41	97.49%	97.43	92.01	87.60
CY S1	6.40	93.88%	97.87	93.68	91.12
CY S2	6.38	93.60%	97.91	93.83	91.71
CY S3	6.35	93.20%	97.96	93.93	90.75
CY S4	6.39	93.73%	97.92	93.82	90.50

3.3. Functional annotation and classification

For functional annotation of the sweet cherry transcriptome, 26,247 unigenes and 14,523 transcripts were aligned with sequences from the reference genome. Of the 26,247 unigenes, about 23,308 (88.80%) unigenes showed homology to the sequences in reference genome. Additionally, 2,939 (11.20%) novel genes were identified in this study. In order to identify new transcript regions, we compared the assembled transcripts with those annotated with the reference sequence, a total of 3,014 (20.76%) novel transcripts were identified. The novel transcripts must meet the following conditions: 200bp or more from the existing annotated transcript and not shorter than 180bp in length (Table 5).

Table 5
Summary of the annotations for the sweet cherry unigenes and transcripts

Items	Number of the unigenes and transcripts	Percentage of annotated unigenes and transcripts (%)
Total genes	26,247	100
Known genes	23,308	88.80
Novel genes	2,939	11.20
Total transcripts	14,523	100
Known transcript	11,509	79.25
Novel transcript	3,014	20.75

Gene Ontology (GO) enrichment analysis was used to classify the function of the DEGs, including biological process (BP), cellular component (CC) and molecular function (MF). In the BP category, a large number of genes were distributed in the cellular process (7388, 11.83%) and metabolic process (5797, 9.28%). As for the CC category, the majority of unigenes were grouped into the cellular anatomical entity (11356, 18.18%) and intracellular (6583, 10.54%). Genes in the MF category were primarily sorted into the binding (8997, 14.41%) and catalytic activity (8630, 13.82%) (Fig. 4A). The DEGs-enriched KEGG pathway scatter plot of JNH vs CY at four different ripening stages were showed in Fig. 4B.

3.4 Differential genes expression

In this study, we screened the differentially expressed genes (DEGs) under the criteria of $|\log_2FC| \geq 1$, and $FDR < 0.05$. We compared the DEGs within a cultivar and between two cultivars within a specific stage, the number of up- and down-regulated unigenes were shown in Fig. 5A. With the fruit ripening, the number of the up- and down-regulated genes between JNH and CY decreased and then increased from stage 1 to stage 4. Of these, the ripening stage 4 possessed the largest number of DEGs: approximately 3,409 up-regulated and 3,222 down-regulated unigenes between JNH4 and CY4. In addition, within the individual cultivars, we found that the number of DEGs between the ripening stage 1 and 4 were significantly higher than any other comparisons.

The DEGs were visually in Venn diagrams to observe the overlap of expression patterns. A total of 12,443 DEGs between the JNH and CY in four different ripening stages were identified, of which 784 (6.30%) genes showed significant differential expression in all the four pairwise comparisons (Fig. 5B).

3.5. Identification of DEGs involved in anthocyanin and carotenoid metabolic pathway

We analyzed the expressions of 11 and 6 structural genes involved in anthocyanin and carotenoid metabolic pathways by RNA-seq (Fig. 6). All of the anthocyanin genes (including *PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS* and *UFGT*) was significantly up-regulated in red fruits JNH (ripening stage 3 and stage 4) in present study. Furthermore, the expressions of anthocyanin genes in JNH began to significantly increased when fruits turning red (ripening stage 3). However, all of the DEGs encoding anthocyanin biosynthesis in CY showed significantly lower levels when fruits turning yellow (ripening stage 3 and 4). Based on the transcriptome data, *CHS* and *UFGT* expressed 1134.58 and 1151.24 times higher levels in JNH compared with CY at stage 4, respectively.

On the other hand, genes (including *PSY*, *PDS*, *HYB*, *LCYB*, *LCYE*) on the pathway of carotenoid biosynthesis, expressed higher levels at stage 1 in two cultivars. Among them, *HYB* and *LCYE* expressed significantly higher levels in CY than that in JNH at stage 1. Interestingly, after stage 1, there was no marked upregulation in the expressions of carotenoid genes (except for *ZEP*) of JNH and CY with ripening. *ZEP* expression was slightly upregulated at stage 3 and stage 4 in both two cultivars.

In addition, a novel *CHS* gene (BGL_novel_G000094) was obtained by RNA-Seq in the present study (Fig. 6), and its expression was basically consistent with that of *CHS* (LOC 110758924) in the reference genome. The FPKM values underlying the heatmap from the transcriptome mapping data were showed in S3 Table.

3.6 Correlation analysis of the DEGs between red and yellow sweet cherries

To further explore the gene expression relationship between red (JNH) and yellow (CY) cherry fruits in anthocyanin and carotenoid biosynthesis, the correlation analysis was conducted. The correlation coefficient (r) ranges between -1 and 1 (Fig. 7). As shown in Fig. 7A, except for *UFGT*, all of the anthocyanin genes in CY were negatively correlated with that in JNH. Contrastly, there were some strong correlations were observed between JNH and CY fruits in carotenoid genes expressions. For example, the expression of *PDS* in CY was significantly related to the expression of *PSY* in JNH during the ripening stages ($r = 0.99$, $p < 0.05$). However, we also found that the expression of *ZEP* in CY was basically negatively correlated with all of the carotenoid genes (except *ZEP*) in JNH (Fig. 7B).

3.5. Validation of RNA-seq data using qRT-PCR

To validate the accuracy of the RNA-seq results, we verified the expression pattern of the 11 genes (*CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS*, *UFGT*, *PDS*, *HYB*, *LCYB*, *LCYE*) in red (JNH) and yellow (CY) cherry fruits during the ripening (Fig. 8). All of the

selected anthocyanin unigenes showed much higher gene expression levels in JNH than CY at ripening stage 3 and stage 4. Additionally, carotenoid structural genes generally expressed lower levels than anthocyanin structural genes expressions in two cultivars. Among them, *HYB* and *LCYE* showed much higher levels in CY compared with JNH at ripening stage 1, which were consistent with the transcriptome data (Fig. 6). Furthermore, the correlation coefficient (*R*) of the expression levels of the 11 genes (*CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS*, *UFGT*, *PDS*, *HYB*, *LCYB*, *LCYE*) were measured by RNA-seq and qRT-PCR were 0.999, 0.984, 0.993, 0.957, 0.999, 0.997, 0.999, 0.644, 0.803, 0.964, 0.841 (Supplementary Fig. S1). Thus, the RNA-seq data here are credible.

4 Discussion

The development cycle of sweet cherry fruit is short and the color changes quickly. Previous studies have shown that the red color of sweet cherry fruit is mainly due to the accumulation of anthocyanin, in which cyanidin-3-*O*-rutinoside (C3R) is the most abundant compound (Ballistrer et al., 2013; Liu et al., 2011). Our results revealed that C3R, as the predominant anthocyanin in red cherries, otherwise, which is significantly lower in yellow cherries. It is indicated that C3R play pivotal role in fruit coloration in red fruit but least in yellow fruits, especially at ripening stage 4 (Table 3). Also, we analyzed the total carotenoid content in red and yellow cherries at four ripening stages and after maturity. The results showed that the content between two cultivars were relatively close during the ripening stages. However, after maturity, the carotenoid content in yellow fruits appeared more stable than that in red fruits. We speculated that this may be due to the down-regulation of anthocyanin genes in yellow cherries after maturity; or may because the genes or transcription factors involved in carotenoid biosynthesis were expressed or regulated more stable in yellow cherries, thereby delaying the degradation of carotenoid in CY. Together, our results suggested that yellow color formation in cherry fruits resulted from insufficient anthocyanin biosynthesis during ripening, but not resulted from the adequate accumulation of carotenoid.

Previous studies have proposed that *CHS*, *F3H*, *DFR* and *ANS* increased gradually with fruit ripening in red cherries, but these genes showed significantly lower expressions in half red and half yellow cherries and always kept at a low level (Liu et al., 2013; Wang et al., 2010). Our results showed that these genes expressed significantly higher levels in red fruits than that in yellow fruits at late ripening stages of sweet cherry (Figure. 6, Supplementary Table 3). However, Liu et al. (2013) have reported that *UFGT* was significantly correlated with bicolored cultivar 'Caihong', and *CHS* was highly correlated with red cultivar 'Hongdeng' by principal component analysis (PCA). These findings were inconsistent with our present results. Our results showed that *CHS* and *UFGT*, expressed 1134.58 and 1151.24 times higher levels in red fruit compared with yellow fruit at stage 4, respectively, indicating that higher expression levels of *CHS* and *UFGT* were tightly linked to the sufficient anthocyanin biosynthesis in the fruits of red cherries, but not to yellow cherries. *CHS* is the first key enzyme in the biosynthesis of flavonoids, and it catalyzes the synthesis of naringenin chalcone from three molecules of 4-malonyl-CoA, and one molecule of p-coumaroyl CoA at the beginning of anthocyanin synthesis (Tanaka et al., 2008). *UFGT* is a key enzyme that catalyzes the transfer of glycosyl from uridine 5'-diphosphoglucose to anthocyanin 3'-*O*-glucosyltransferase at the last step of anthocyanin biosynthesis, which is to convert unstable cyanidin into stable anthocyanin (Forkmann and Heller, 1999; Kubo et al., 2007). Studies have reported that *UFGT* is closely related to anthocyanin biosynthesis in the developmental stage of red apple, red sand pear and grape fruit (Boss, Davies and Robinson, 1996; Feng et al., 2008; Liu et al., 2012). From our results, we get to know that *UFGT*, one of the structural genes on anthocyanin biosynthetic pathway, was the most differentially expressed gene between red and yellow varieties, which suggested to be the key gene caused color difference between red and yellow fruits in mature cherry fruits. This may be because *UFGT* could catalyze the attachment of a sugar to the anthocyanin aglycone more quickly and more significant in red compared with yellow fruits, considerably increasing its stability.

Meanwhile, a previous study has shown some regulatory unigenes in the anthocyanin biosynthesis of the red and yellow fruits of sweet cherry, but not in the carotenoid biosynthesis (Wei et al., 2015). Here, we analyzed anthocyanin and carotenoid biosynthetic gene expression levels based on the pigmental substances and different ripening stages in sweet cherry fruits. We found that all the structural genes involved in carotenoid biosynthesis showed positive correlations between JNH and CY, except *ZEP* (Figure. 7B). It indicated that the expression pattern of carotenoid biosynthetic genes in JNH and CY were relatively similar during the ripening. *ZEP* is an enzyme that catalyzes zeaxanthin to form antherxanthin, and then catalyze antherxanthin to form violaxanthin, which was slightly up-regulated in two cultivars at S3 and S4 in present study, suggesting that violaxanthin content in red and yellow cherries would relatively increase at the late ripening stage. More importantly, β -carotene hydroxylase (*HYB*) was a rate limiting enzyme that catalyzes the formation of zeaxanthin. *HYB* was the unigene that encoded *HYB* formation. *HYB* peaked the highest expression level at S1 and then decreased in late ripening stages in both of red and yellow cherries (Alquezar, Rodrigo and Zacarías, 2008). Especially in yellow cherries, the expression of *HYB* decreased by 65.47% from S1 to S4 (Fig. 6, Supplementary Table 3). On the one hand, this could be due to the interactions of other genes that inhibited *HYB* expression at the late ripening stage; on the other hand, the insufficient catalytic substrate in upstream of *HYB* could be another reason for it. Additionally, in yellow cherry fruits, *HYB* expressed a 1.66 times higher level compared with red fruit at S1, we supposed that *HYB* may enhance a flux from β , β -ring hydroxylation to β , ϵ -ring hydroxylation, which may result in an increase of zeaxanthin in red fruits at early stage of ripening (Fig. 9). For this result, the changes of specific carotenoid components need to be further explored. In summary, the imbalanced regulation of *HYB* and *ZEP* and gene-interactions could be the reason for the different color formation in red and yellow cherry fruits in this study.

The findings presented in this study offer valuable insights into the transcriptional regulation of color biosynthesis during ripening among the different *P. avium* varieties. The discrepant pigmental substance of the color change in red and yellow fruits during ripening stages were identified. Numerous It is also recommended to conduct the more metabolome profiling (including primary and secondary substances) among the different varieties to systematically evaluated the metabolite differences for fruit coloration for possible functional biomarker development.

Conclusion

We have performed a combined analysis of transcriptome and metabolome of the red (JNH) and yellow (CY) sweet cherry at the four ripening stages. The profiles of anthocyanin and total carotenoid derived from the red and yellow fruits were identified, the differential expression genes (DEGs) on the anthocyanin and carotenoid biosynthetic pathways were systematically compared. As for the pigmental metabolites, cyanidin-3-*O*-rutinoside (C3R) was the most different pigments between red and yellow cherry fruits, with a significantly higher content in red fruits than that in yellow fruits; the total carotenoid contents between two cultivars were relatively close in value, but the contents in yellow fruits showed more stable than that in red fruits after maturity. The expressions of *CHS* and *UFGT* in the red fruits were significantly higher than those of yellow fruits at late stages of ripening; but the expressions of structural genes in carotenoid biosynthetic pathway showed little difference between red and yellow cherries.

Declarations

Author contributions

Wangshu Zhang designed this work and substantively revised the manuscript. Qinghao Wang drafted the manuscript and contributed to the data curation. Luyang Jing and Yue Xu performed the experiments and analyzed the data. Weiwei Zheng contributed to the technical support.

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Availability of data and materials

All data generated or analysed during this study are included in the supplementary information files. The raw RNA-seq data are freely available at <https://www.ncbi.nlm.nih.gov/sra/PRJNA905870>

Compliance with ethical standards

There are no ethical issues involved on this work. Experimentation with living animals did not happen in this work.

Conflict of Interest

Qinghao Wang declares that he has no conflict of interest. Luyang Jing declares that she has no conflict of interest. Yue Xu declares that he has no conflict of interest. Weiwei Zheng declares that he has no conflict of interest. Wangshu Zhang declares that he has no conflict of interest.

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Figures

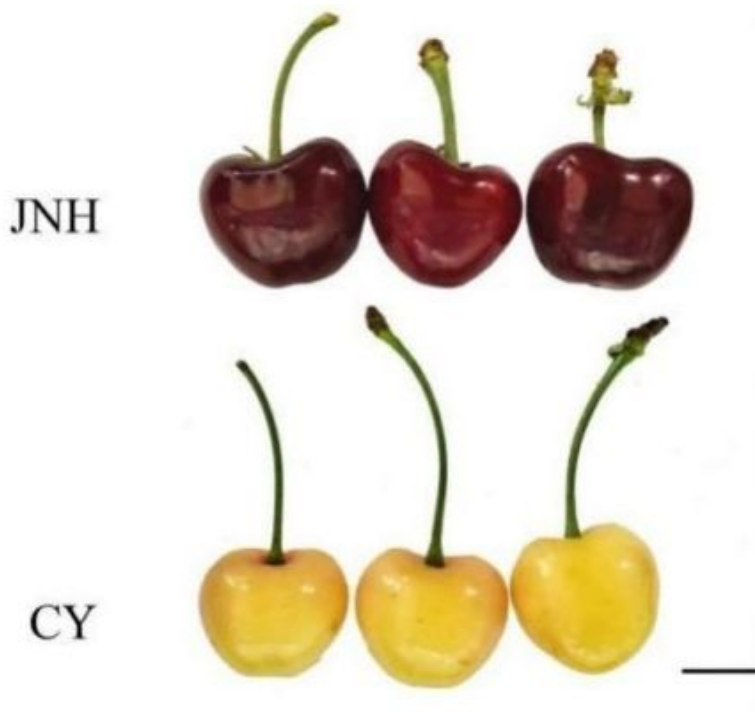


Figure 1

Fruit morphology of JNH and CY are shown. The bars of the sections are 1.0 cm.

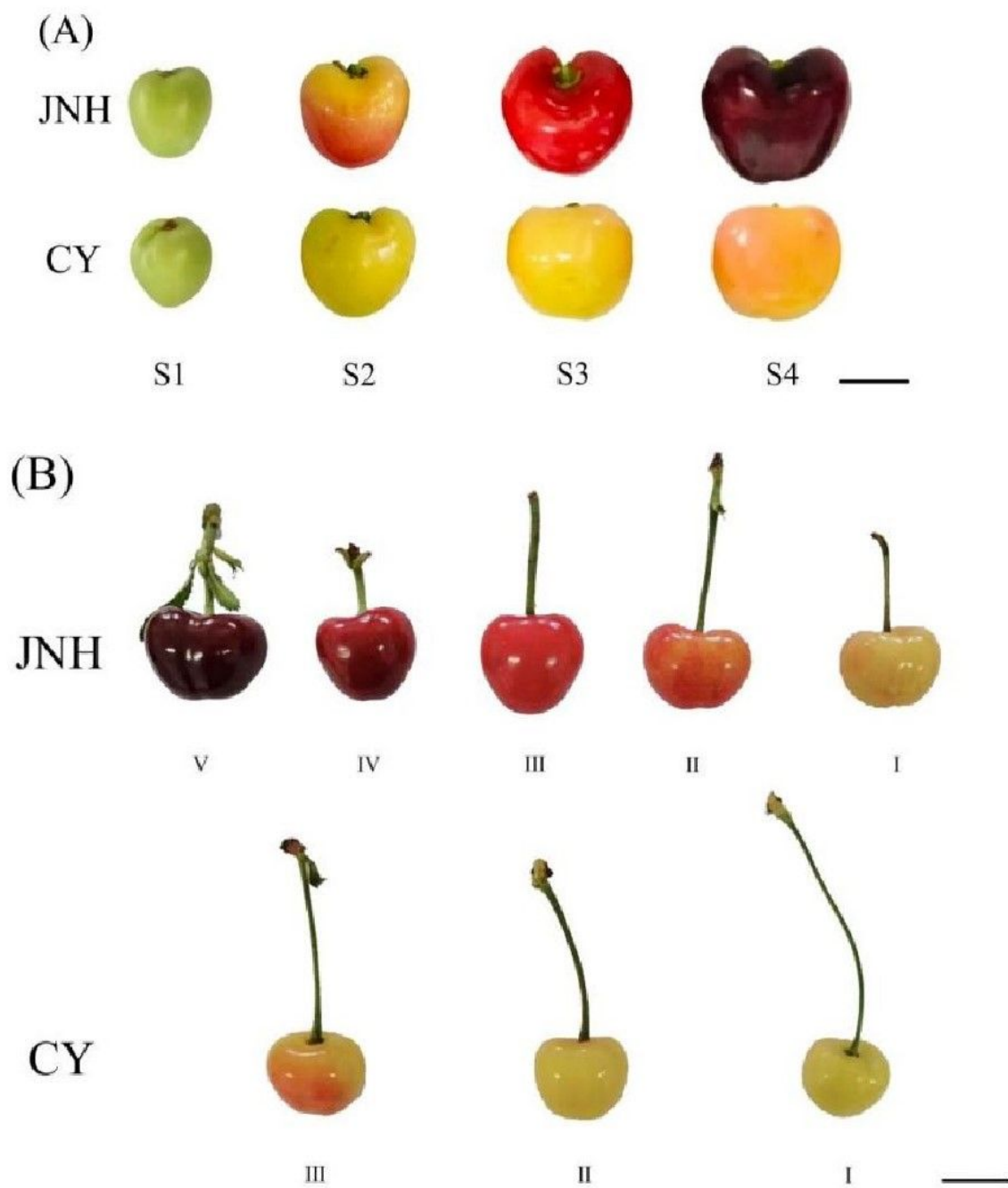


Figure 2

Different ripening stages (A) and maturity level (B) of JNH and CY were showed. The bars of the sections are both 1.0 cm.

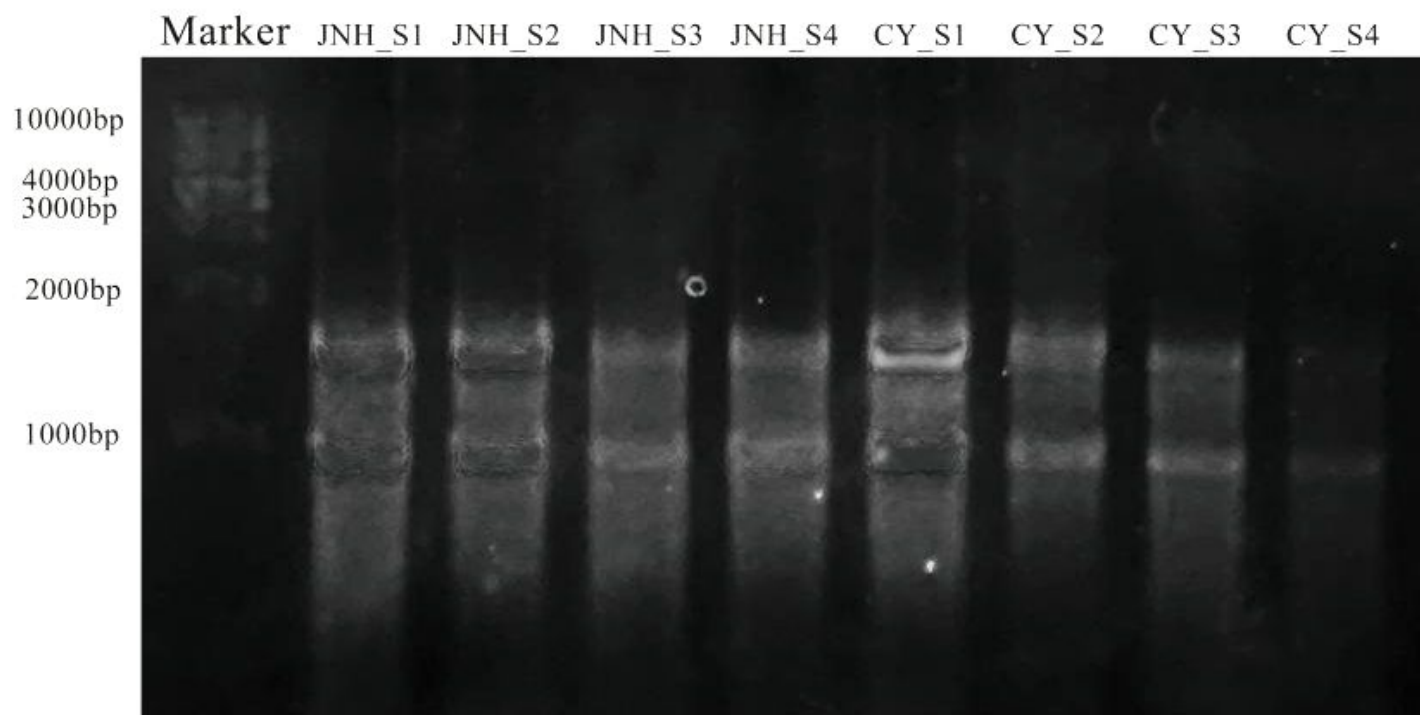
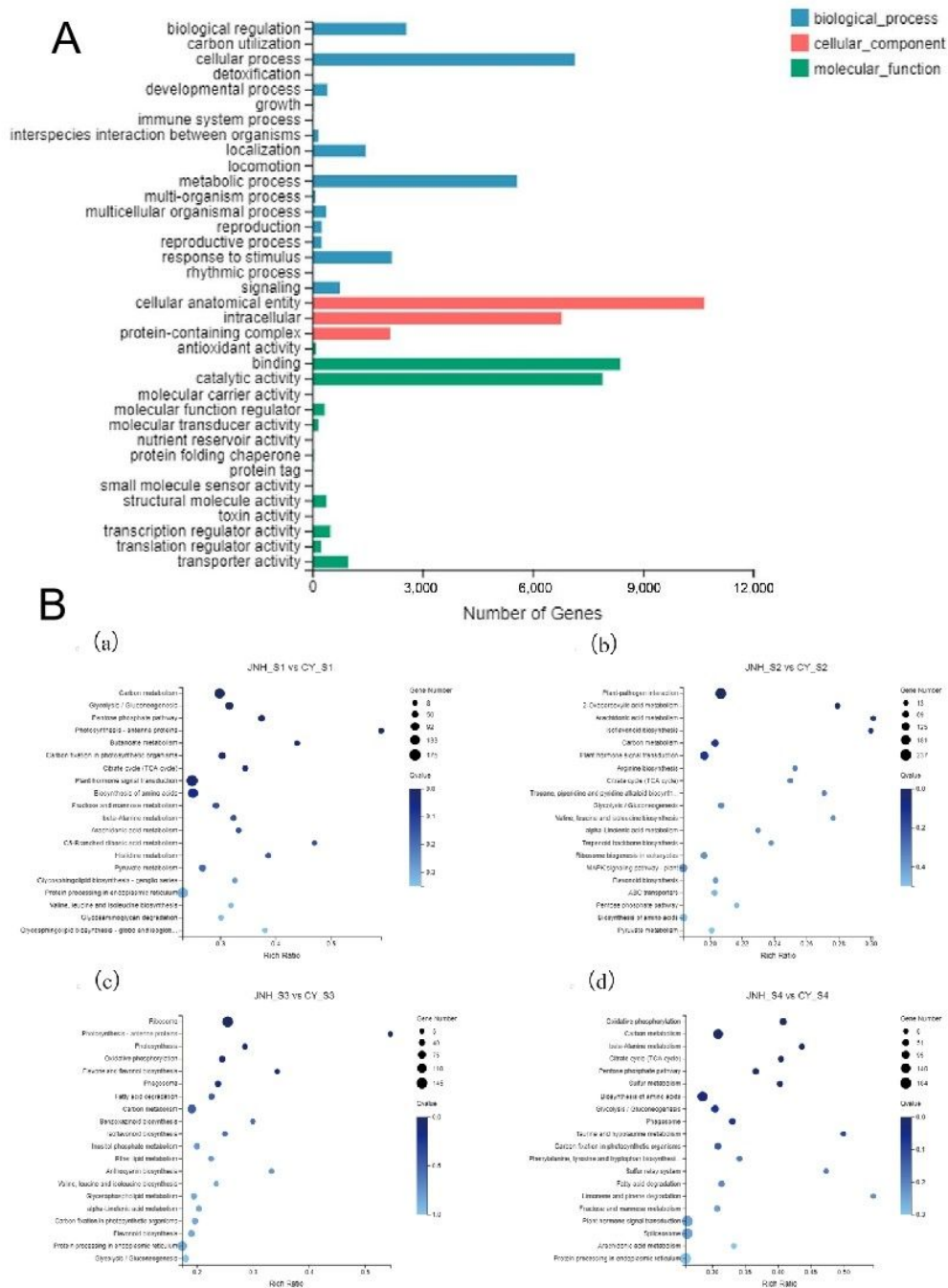


Figure 3

The agarose gel electrophoresis (1.5%) testing of the integrity of RNA.



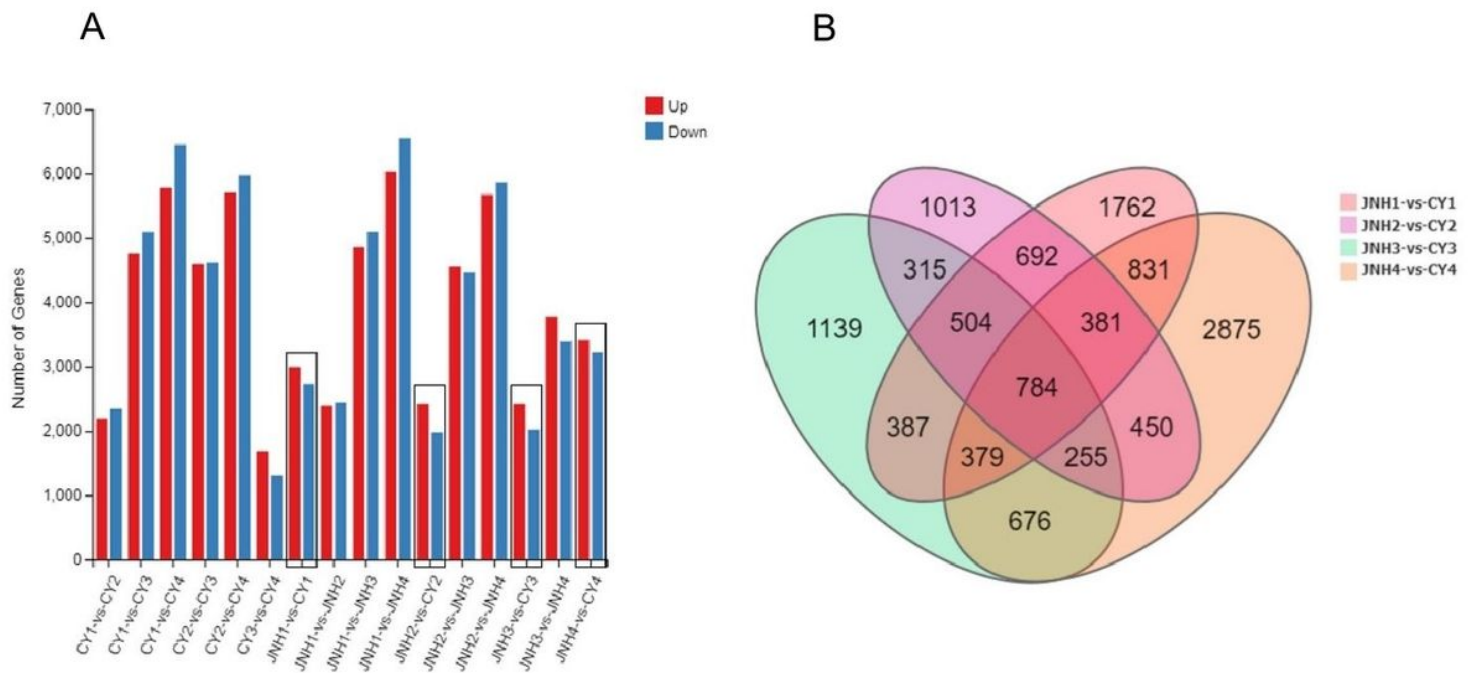


Figure 5

Overview of the differentially expressed genes (DEGs) of JNH and CY fruits at four different ripening stages. (A). Number of DEGs between different stages of fruit and between cultivars identified by pairwise comparison ($\log^2$ Fold Change ≥ 1 and adjusted FDR < 0.05). The bar chart framed in black boxes were the DEGs between JNH and CY in four specific ripening stages. (B). A venn diagram showed the numbers of the DEGs in four pairwise comparisons.

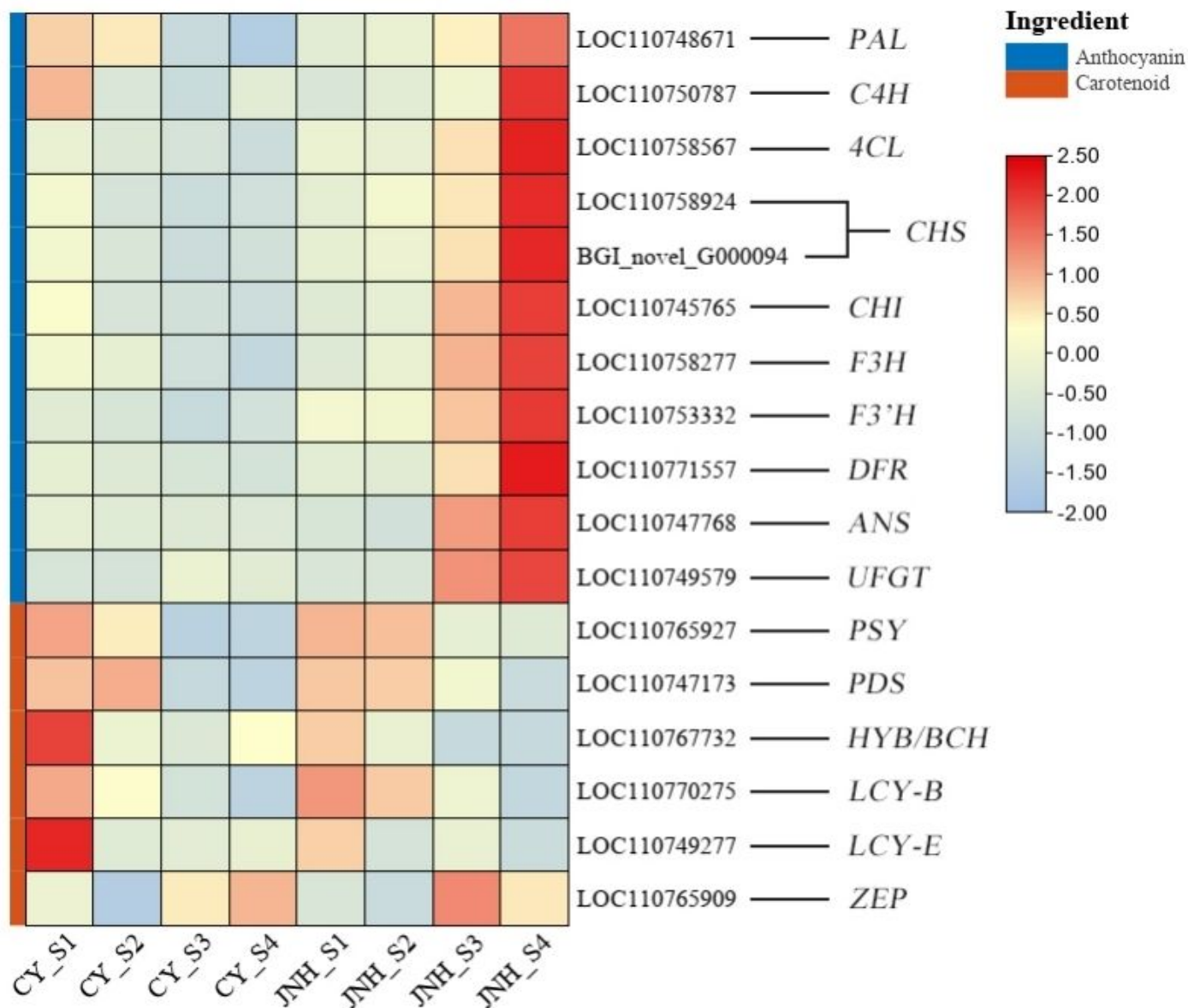


Figure 6

Visualizing heatmap of structural gene expression of anthocyanin and carotenoid in the fruit of sweet cherry. S1, S2, S3, S4 refers to the four different ripening stages of JNH and CY. The underlying transcriptome mapping data are presented in **S3 Table**.

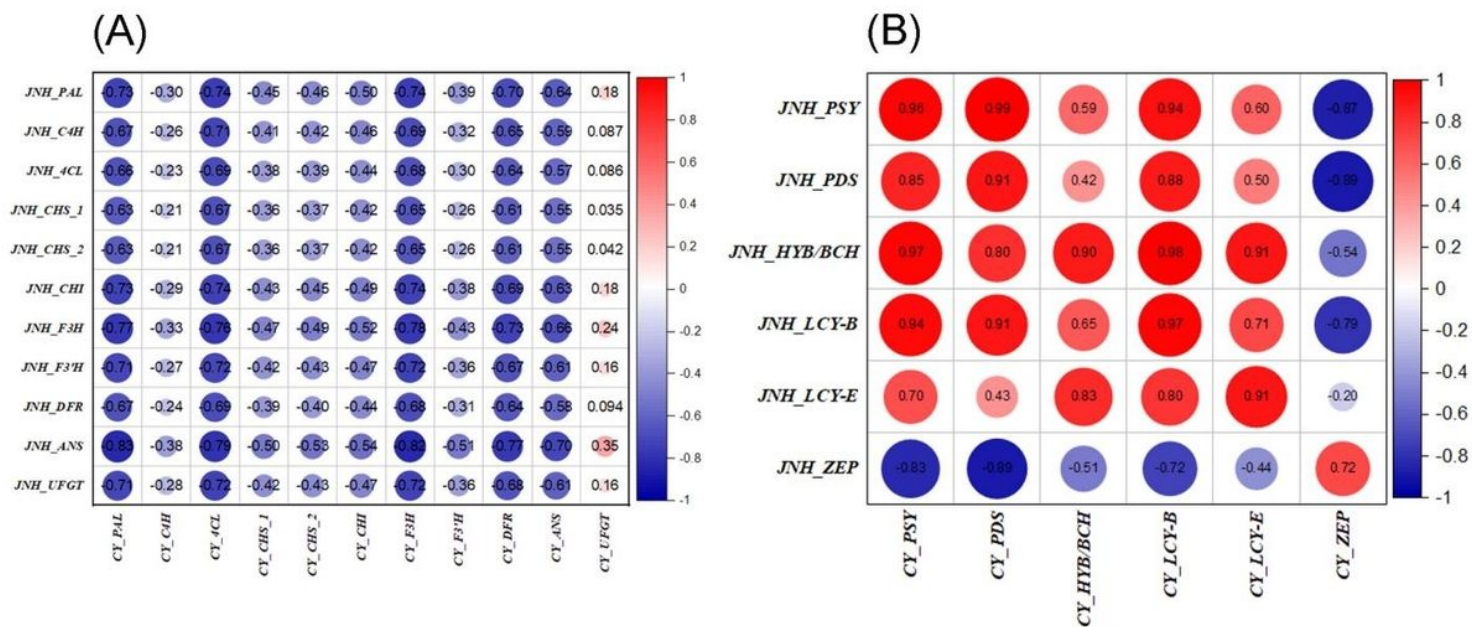


Figure 7

Correlation analysis of anthocyanin (A) and carotenoid (B) genes between JNH and CY fruits from the RNA-seq data. Red indicates positive correlation and blue indicates negative correlation ($p < 0.05$).

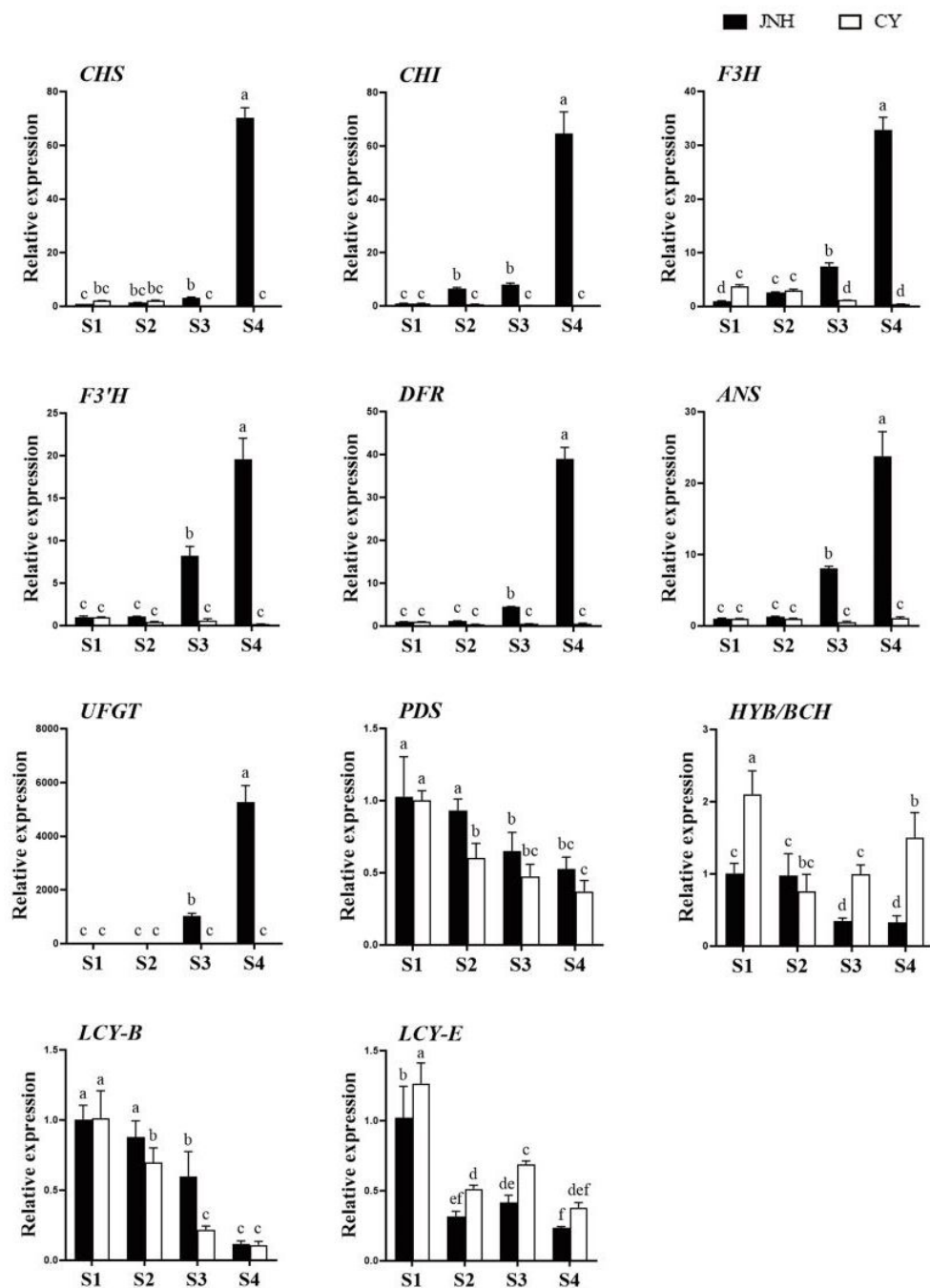


Figure 8

Expression analysis of eleven genes related to anthocyanin and carotenoid biosynthesis in sweet cherry using qRT-PCR. The standard error of the mean for three biological replicates (nested with three technical replicates) is represented by the error bars ($p < 0.05$).

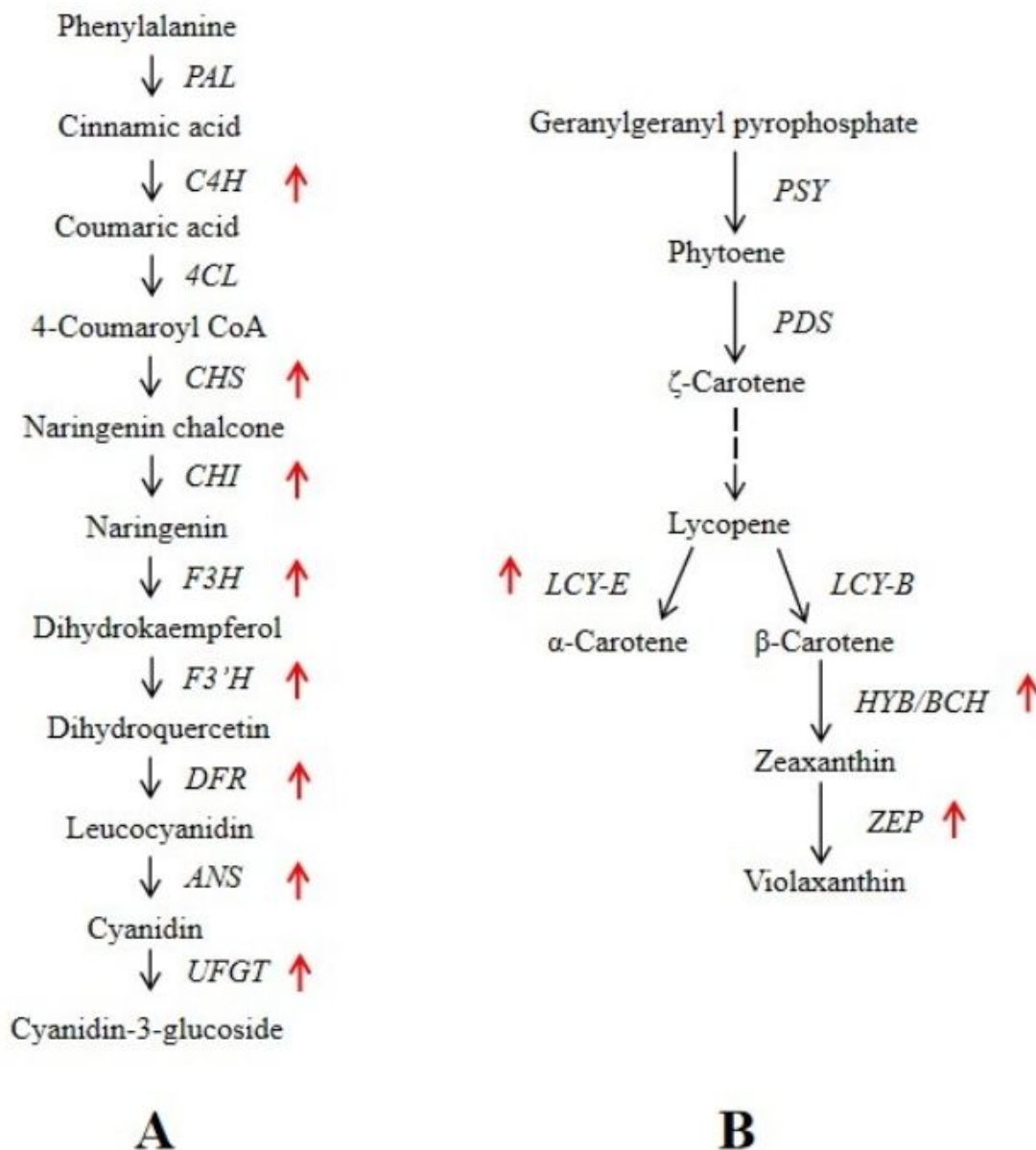


Figure 9

Relative pathway and enzymes involved in anthocyanin (A) and carotenoid (B) biosynthesis of *P. avium* fruit. The up arrow in anthocyanin pathway indicates JNH S4 vs CY S4 upregulated. The up arrow in carotenoid pathway indicates CY S1 vs JNH S1 upregulated.

Supplementary Files

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