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Integrated Analysis of Transcriptome and Metabolome Reveals New Insights into the Molecular Mechanism Underlying the Color Differences in Wolfberry (*Lycium barbarum*)

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Abstract: Wolfberry (*Lycium barbarum* L.) is a small Solanaceae shrub with medicinal and edible homology, and widely used as ethnobotanical medicine and nutraceutical food. The wolfberry fruits mainly have red, purple, and yellow phenotypes. Wolfberries are rich in flavonoids, which are natural water-soluble pigments that endow a variety of colors in plants. There are very few investigations on mechanism of flavonoids biosynthesis and fruit coloring reported about wolfberry. The widely targeted metabolome and transcriptome analysis were performed to obtain metabolite and gene expression profiles of red, yellow, and purple wolfberries and to explain the underlying molecular mechanism of the color differences in wolfberry. As result, metabolomics analysis revealed that the bluish anthocyanins Malvidin and petunidin trended to accumulate in purple wolf-berry, while red and yellow wolfberries trended to accumulate more yellowish flavonoids. And transcriptome analysis showed that flavonoid synthesis-related genes, such as *CHS*, *F3H*, *ANS* and *DFR*, and several *MYB* and *bHLH* genes were differentially expressed among wolfberries in different colors: most of them were more highly expressed in purple wolfberries than in red and yellow ones. In conclusion, the different flavonoids' accumulation patterns may result in the different fruit colors of wolfberry, and the MYB or bHLH transcription factors could regulate the expression of flavonoids biosynthesis related genes to change the composition of flavonoids or anthocyanins in wolfberry fruits and result in varied fruit colors. These findings provide new insights into the underlying molecular mechanism of the fruit color differences in wolfberry and provide new ideas for molecular breeding of wolfberry.

Keywords: metabolomics; transcriptomics; flavonoid biosynthesis; anthocyanin biosynthesis



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1. Introduction

Wolfberry (*Lycium barbarum* L.) is a small Solanaceae shrub with medicinal and edible homology, and widely used as ethnobotanical medicine and nutraceutical food. It has been implicated in replenishing vital essence, improving eyesight, and nourishing the liver and kidneys [1,2]. Pharmacological studies of wolfberry compounds have shown many beneficial uses. For instance, *L. barbarum* polysaccharides (LBPs), unique, water-soluble glycoconjugates in wolfberry, possess antiaging, antidiabetic, antifibrotic, neuroprotective, and immunomodulating properties [3]. Furthermore, polyphenols such as flavonols, anthocyanins, and catechins have been found to be strong natural antioxidants [1].

Color has been used as a quality evaluation index in traditional Chinese medicine and is related to its intrinsic material base. Common plant pigments include chlorophyll, carotenoid, beet pigment, and flavonoid pigment, the physical and chemical properties of which, as well as mutual mixing and modulation, create colorful color characteristics of plants. Anthocyanins, a subgroup of flavonoids, are natural, water-soluble pigments that endow a variety of colors in plants [4–6]. Six main anthocyanin pigments in plants, such

as cyanidin, delphinidin, pelargonin, peonidin, malvidin, and petunidin, show different colors [7,8]. Thus, the anthocyanin biosynthesis pathway is related to color phenotype [4–6].

Currently, much is known about the core flavonoid biosynthesis pathway. Firstly, the phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), and 4-coumarate CoA ligase (4CL) convert phenylalanine to coumaroyl-CoA. Secondly, coumaroyl-CoA and malonyl-CoA are synthesized into dihydroflavonol with catalyticases such as chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3O-hydroxylase (F3OH), flavonol synthase (FLS), and flavone synthase (FNS). Thirdly, dihydroflavonol forms leucoanthocyanidins via the action of dihydroflavonol 4-reductase (DFR), and then anthocyanidins are synthesized by anthocyanidin synthase (ANS). Finally, the anthocyanidins are modified by flavonoid glucosyltransferase (UGT) and anthocyanin O-methyltransferase (AOMT) [9–11].

The integrated analysis of transcriptomics and metabolomics can comprehensively reflect the nature of sample phenotypic differences by comparing the differences at the gene and metabolite level of samples with different phenotypes, combining the relationship between the expression changes of these two substances [12,13], and have been widely used to study the synthesis and regulation mechanisms of metabolites in many plants [5,6], such as tomato fruits [14], watermelon fruits [15], peach fruits [16], and sugarcane [17]. There have been limited investigations on the regulatory mechanisms of anthocyanin synthesis of red, yellow, and purple fruit colors in wolfberry [18].

Hence, in order to determine the composition patterns of flavonoid compounds and the flavonoid-synthesis-related gene expression profiles in wolfberry, widely targeted metabolome and transcriptome analysis of red, yellow, and purple wolfberries was performed and integrated. It is vital for exploring the relationship between gene expression, metabolite accumulation and color formation, and explaining the underlying molecular mechanism of the color differences in wolfberry. And the results would provide new ideas and a new theoretical basis for the molecular breeding of wolfberry.

2. Materials and Methods

2.1. Plant Materials

The fruits of the red wolfberry cultivar, Ningqi7, yellow wolfberry cultivar, W-12-27, and purple wolfberry cultivar, ZH-13-01, were collected from the germplasm resource nursery of *Lycium barbarum* in Luhutai Garden Farm, Yinchuan City, Ningxia in 2019. The matured wolfberry fruits in different colors were frozen in liquid nitrogen, and then stored at -80°C for subsequent metabolome and transcriptome analysis. All experimental samples were repeated three times.

2.2. Metabolites Extraction

About 100 mg of the frozen fruit tissue was crushed with a zirconia bead in a mixer mill (MM 400, Retsch) for 1.5 min at 30 Hz and extracted with 0.6 mL 70% aqueous methanol for overnight at 4°C . Prior to LC-MS analysis, the extracts were centrifugated at $10,000\times g$ for 10 min and then filtrated using microporous membranes (SCAA-104, $0.22\text{ }\mu\text{m}$ pore size; ANPEL, Shanghai, China).

2.3. LC-ESI-MS/MS Analysis

The HPLC system (Shim-pack UFLC SHIMADZU CBM30A) with Waters ACQUITY UPLC HSS T3 C18 HPLC column ($1.8\text{ }\mu\text{m}$, $2.1\text{ mm}\times 100\text{ mm}$) and the LC-ESI-MS/MS system (Applied Biosystems 4500 Q TRAP) were utilized for metabolomics analysis of the sample extracts. And the mobile phase solvent A was 0.1% formic acid in water and solvent B was acetonitrile with 0.1% formic acid. The chromatography flow rate was $350\text{ }\mu\text{L}/\text{min}$ and a linear gradient was programmed for 14 min: 95%A:5%B (*v/v*) at 0 min, 5%A:95%B at 9.0 min, 5%A:95%B at 10.0 min, 95%A:5%B at 11.1 min and kept for 2.9 min. And the column oven temperature was 40°C and the injection volume was $2\text{ }\mu\text{L}$ [19].

Linear ion trap (LIT) and triple quadrupole (QQQ) scans were obtained with a triple quadrupole-linear ion trap mass spectrometer (Q TRAP), API 4500 Q TRAP LC/MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled via Analyst 1.6.3 software (AB Sciex) [19]. The ESI source operation parameters were as follows: ion source, turbo spray; source temperature 550 °C; ion spray voltage (IS) 5500 V (positive ion mode)/−4500 V (negative ion mode); ion source gas I, gas II, and curtain gas were set at 50, 60, and 30.0 psi, respectively [19]. The collision gas was high. Instrument tuning and mass calibration were performed with 10 and 100 µmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were obtained as MRM experiments with collision gas (nitrogen) set to 5 psi [19]. DP and CE for individual MRM transitions was done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period [19].

2.4. Metabolite Data Analysis

Before metabolite data analysis, a quality control (QC) analysis was conducted to confirm the reliability of the data. The QC sample, the mixture of all sample extracts, was used to monitor the changes in repeated analyses. Metabolite identification was performed basing on the Metware MWDB database, following their standard metabolic operating procedures [19]. Sciex analyst work station software Version 1.6.3 (Sciex, Framingham, MA, USA) was employed for multiple-reaction-monitoring (MRM) metabolite quantification [20]. The principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and 200 permutation tests for PLS-DA were performed to obtain the variable importance in projection (VIP). Metabolites with $VIP \geq 1$ and fold change ≥ 2 or fold change ≤ 0.5 were considered as the significantly differentially accumulated metabolites (DAM). Additionally, the Pearson correlation analysis and hierarchical clustering heatmap were performed with R software version 4.2.1 (www.r-project.org, accessed on 21 September 2022).

2.5. Transcriptome Sequencing and Data Analysis

The TRIzol reagent (Invitrogen, Waltham, CA, USA) was used to extract the total RNA of samples according to the manufacturer's instructions. The RNase-Free DNase (Promega, Madison, WI, USA) was used to remove genomic DNA in the RNA extracts. The concentration and purification of the RNA was estimated via Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), and then the RNA quality was confirmed via 1% agarose gel electrophoresis. The cDNA libraries were constructed with the eligible RNA extracts. Then the high-throughput sequencing was carried out in Metware Biotechnology Co., Ltd. (Wuhan, China) on the Illumina HiSeq™ 2500 platform (Illumina Inc., San Diego, CA, USA) [15]. Subsequently, after removing the adaptors and the low-quality reads, the clean reads were obtained and then mapped to the reference *Lycium barbarum* genome sequence [21] via HISAT2 v2.1.0. And the gene expression quantification was performed based on the fragments per kilobase of exon model per million mapped fragments (FPKM) using featureCounts v1.6.1. The DESeq2 (version 1.40.2) package was used to perform the differential gene expression analysis with R version 4.2.1 [22]. The genes whose fold change was ≥ 2 or ≤ 0.5 , and whose Student's T test *p* value was <0.05 and whose Q value was <0.05 were regarded as the differentially expressed genes (DEGs). The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway functional enrichment analyses of DEGs were performed, respectively. Fisher's exact test was used to evaluate the significance of the GO and KEGG enrichment analyses.

3. Results

3.1. Transcriptome and Metabolome Characteristics of *L. barbarum* Fruits Colors

To better understand the molecular basis of yellow, red, and purple fruit colors in *L. barbarum*, fruit samples were collected (Figure 1), and their metabolomes and transcriptomes were analyzed using widely targeted metabolomics and high-throughput RNA-sequencing technologies. Based on these results, principal component analysis (PCA) and Pearson correlation analysis were performed. The PCA results showed that the yellow, red, and purple fruits were clustered in different PCA spaces, both in gene expression level and metabolite abundance level, indicating that the differently colored *L. barbarum* fruits had significant differences in gene expression and metabolite accumulation. Thus, the molecular characteristics may be responsible for the different color phenotypes (Figure 2A). The Pearson correlation analysis showed that the transcriptome correlation between yellow and purple fruits was higher than that between red and yellow or purple fruits. Although the metabolome PCA showed that the different colors were distinguishable, the correlation between their metabolome was high, indicating robust reproducibility of the metabolomics and transcriptomic experiments (Figure 2A).

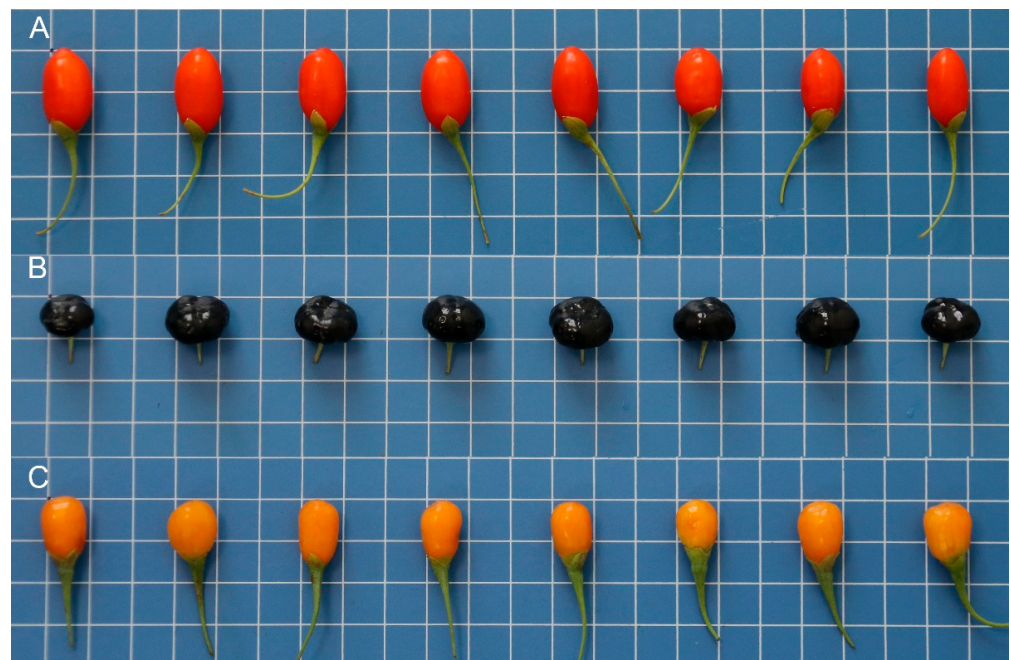


Figure 1. Fruits of the yellow, red and purple wolfberry. (A–C) are the red, purple, and yellow wolfberry fruits, respectively.

3.2. Differentially Expressed Genes among Yellow, Red, and Purple *Lycium barbarum* Fruits

The transcriptome sequencing of yellow, red, and purple *L. barbarum* fruits generated 64.63 Gb of clean reads after eliminating the adaptor sequences and low-quality reads. The average percentage of the high-quality score (Q30) was 97.45%, the GC contents were 42.5%, and the average mapped ratio of clean reads was 89.66% (Table 1). There were 5788 (1889 up-regulated, 2146 down-regulated), 4462 (2614 up-regulated, 3174 down-regulated), and 4035 (1947 up-regulated, 2515 down-regulated) differentially expressed genes (DEGs) that were screened out in the criterion whose FC was >2 and *p*-value was <0.05 in R_vs._Y, P_vs._Y, and R_vs._P comparison groups, respectively (Table S1; Figure 3A). Among these comparison groups, there were 752 common DEGs (Figure 3B). Furthermore, we found that some genes of the enzymes involved in the core flavonoid biosynthesis pathway were differentially expressed between the three different wolfberries, such as phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), flavonoid 3-glucosyl transferase (UFGT), 4-coumarate—CoA ligase

(4CL), anthocyanidin synthase (ANS), and anthocyanin O-methyltransferase (AOMT) (Table 2). Most of PAL, CHS, CHI, F3H, UFGT, ANS, and AOMT genes were more highly expressed in purple wolfberries than in red and yellow wolfberries (Table 2). Meanwhile, a lot of transcription-factor-coding genes were found to be differentially expressed between the three different wolfberries, like MYB and bHLH. As shown in Table 2, we found that most of the MYB and bHLH genes' expression levels were higher in purple wolfberries than that in red and yellow wolfberries (Table 2).

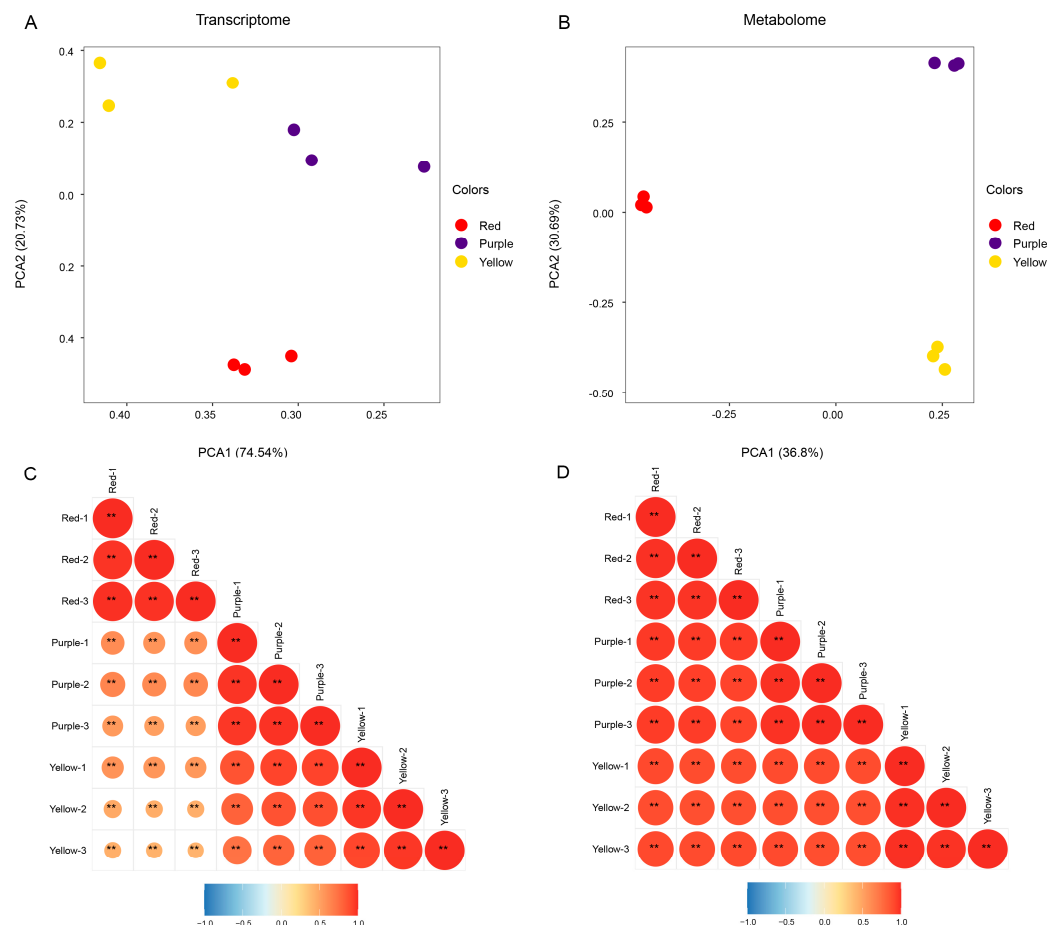


Figure 2. Transcriptome and metabolome characteristics of yellow, red and purple wolfberry fruits. (A,B) Are the PCA results of transcriptome and metabolome, respectively. Red, purple and yellow fruit samples in red, purple and yellow, respectively. (C,D) Are the Pearson correlation analysis results of transcriptome and metabolome, respectively. “**” refer to Pearson correlation coefficients significance levels <0.01 and <0.001, respectively. The scale color from blue to red indicates the Pearson correlation coefficients from small to large.

Table 1. Summary of transcriptomic data.

Sample	Raw Read	Clean Read	Q20%	Q30%	GC%
Red_1	63,079,672	49,752,022	99.98	97.23	42.50
Red_2	56,297,936	42,361,522	99.98	97.13	42.50
Red_3	63,970,362	44,320,914	99.98	97.70	42.50
Purple_1	53,299,500	51,616,046	99.97	97.47	42.50
Purple_2	60,853,292	59,079,736	99.97	97.78	42.50
Purple_3	68,764,014	50,181,666	99.97	97.47	42.50
Yellow_1	53,716,626	41,536,442	99.95	97.29	42.50
Yellow_2	56,895,446	38,520,522	99.95	97.40	42.50
Yellow_3	67,313,950	53,530,618	99.96	97.60	42.50

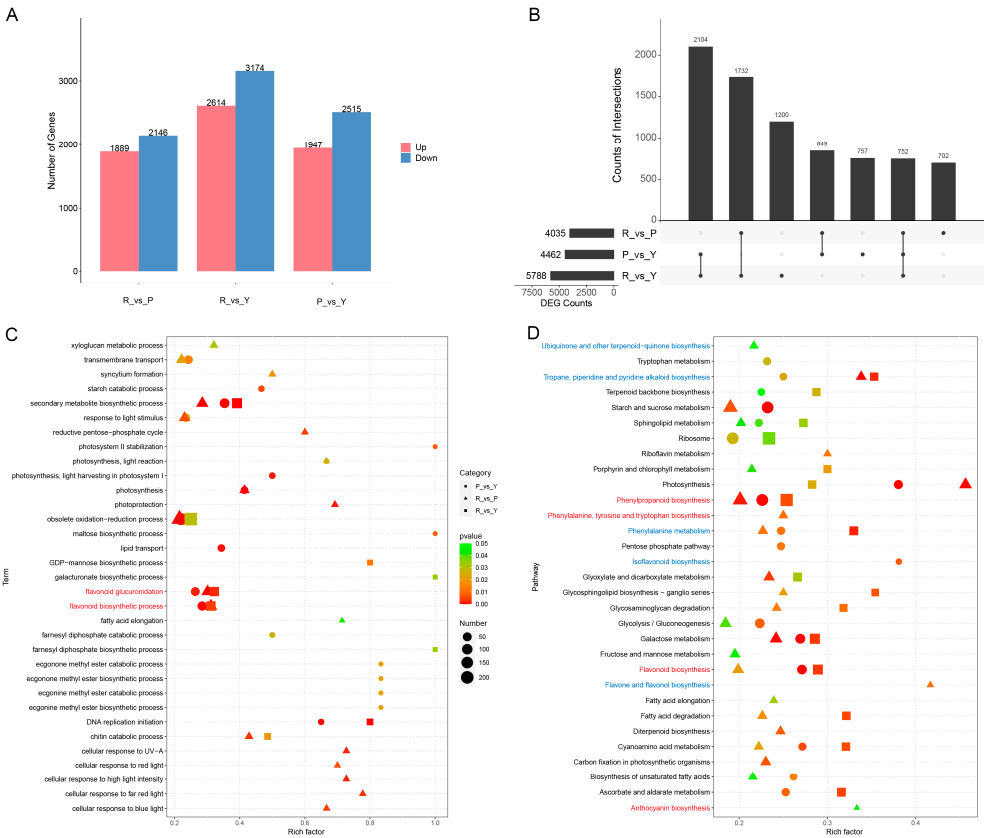


Figure 3. Statistical and functional enrichment analysis of differentially expressed genes. **(A)** The barplot of DEGs among yellow, red, and purple wolfberries. **(B)** The upset plot of DEGs. **(C)** The babbler plot of GO biological process enrichment analysis; the circle, triangle, and square stands for R_vs_Y, R_vs_P and P_vs_Y comparison groups, respectively. **(D)** The babbler plot of KEGG enrichment analysis; the circle, triangle and square stands for R_vs_Y, R_vs_P and P_vs_Y comparison groups, respectively.

Table 2. The DEGs of key enzymes of the core flavonoid biosynthesis pathway and related transcription factors.

	Gene ID	Gene Name	Description	FPKM Expression Mean		
				Red	Purple	Yellow
Enzymes	CCG041018	ANS	anthocyanidin synthase	2.22	995.04	4.40
	CCG036272	AOMT	anthocyanin O-methyltransferase	0.13	1863.70	6.25
	CCG005771	CHI	chalcone isomerase	5.41	310.74	7.38
	CCG033659	F3H	flavanone 3-hydroxylase	72.23	3110.26	129.96
	CCG048821	CHI	chalcone isomerase	2.51	100.35	4.05
	CCG011047	CHS	chalcone synthase, partial	0.10	109.61	2.18
	CCG025288	CHS	chalcone synthase, partial	7.20	657.56	27.33
	CCG040770	4CL	4-coumarate-CoA ligase 2	4.69	27.16	3.91
	CCG014669	PAL	phenylalanine ammonia-lyase	2.37	46.59	6.15
	CCG027374	4CL	4-coumarate-CoA ligase 1	0.06	1.13	0.15
	CCG021038	UFGT	flavonoid 3-glucosyl transferase precursor	8.34	235.24	23.76
	CCG032840	4CL	4-coumarate-CoA ligase 1	10.37	21.27	38.65
	CCG021591	4CL	4-coumarate-CoA ligase-like 6 isoform X2	2.24	4.49	4.79
	CCG042047	4CL	4-coumarate-CoA ligase 2	1.31	1.42	3.34
	CCG019697	F3H	flavonol synthase/flavanone 3-hydroxylase-like	6.88	16.41	31.92
	CCG014668	PAL	phenylalanine ammonia-lyase	2.33	5.94	3.13
	CCG019697	FLS	flavonol synthase/flavanone 3-hydroxylase-like	6.88	16.41	31.92
	CCG028824	DFR	dihydroflavonol-4-reductase	0.42	528.47	2.07
	CCG031097	ANR	anthocyanidin reductase-like	4.06	9.64	7.16

Table 2. Cont.

	Gene ID	Gene Name	Description	FPKM Expression Mean		
				Red	Purple	Yellow
Transcription factors	CCG024292	MYB	MYB1	0.37	139.59	1.16
	CCG005510	MYB	MYBST1 protein	59.06	264.58	161.73
	CCG031911	MYB	transcription factor MYB1R1	33.73	122.07	79.18
	CCG044025	MYB	transcription factor MYB78-like	4.99	1.37	6.19
	CCG047101	MYB	transcription factor MYB48-like	2.89	9.27	6.70
	CCG015627	MYB	transcription factor MYB48-like	0.97	3.61	6.02
	CCG037979	MYB	Transcription factor MYB39	0.07	0.82	0.62
	CCG043970	MYB	transcription factor MYB108-like	1.91	0.76	3.70
	CCG028531	MYB	transcription factor MYB41-like	0.78	1.80	0.36
	CCG030977	MYB	transcription factor MYB101-like	0.36	0.95	0.98
	CCG028535	MYB	transcription factor MYB41-like	0.52	1.22	0.37
	CCG036054	bHLH	transcription factor bHLH66	1.56	28.54	18.99
	CCG028743	bHLH	transcription factor bHLH13-like	1.15	9.39	2.30
	CCG033626	bHLH	transcription factor bHLH36-like	0.00	1.04	0.05
	CCG014150	bHLH	transcription factor bHLH94-like	0.48	4.94	1.51
	CCG011083	bHLH	transcription factor bHLH148-like	61.29	168.73	50.65
	CCG042659	bHLH	transcription factor bHLH137-like	4.25	14.98	7.53
	CCG050226	bHLH	transcription factor bHLH153-like	28.62	60.52	70.14
	CCG041157	bHLH	transcription factor bHLH62-like	4.69	9.49	1.93
	CCG033109	bHLH	transcription factor bHLH30-like	0.32	1.90	5.21
	CCG047551	bHLH	transcription factor bHLH117	0.99	3.37	6.48

3.3. Functional Enrichment Analysis of the DEGs among Yellow, Red, and Purple Wolfberry Fruits

In order to clarify the functional category of DEGs and the biological pathways involved, all DEGs were annotated with the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and functional enrichment analysis was performed (Figure 3C,D; Tables S2 and S3). The GO enrichment results showed that the DEGs of the R_vs._Y, R_vs._P, and P_vs._Y comparison groups were commonly enriched in the secondary metabolite biosynthetic process, heat response, obsolete oxidation—reduction process, and flavonoid-associated biological processes such as flavonoid glucuronidation. The DEGs of P_vs._Y comparison groups were specifically enriched in toxin catabolic processes, starch catabolic processes, response to oxidative stress, reactive oxygen species metabolic processes, glutathione metabolic processes, photosystem II stabilization (photosynthesis light harvesting unit in photosystem I), and lipid transport. The R_vs._P DEGs were specifically enriched in xyloglucan metabolic processes, syncytium formation, response to Karrikin, reductive pentose—phosphate cycle, photoprotection, cellular response to UV-A, cellular response to red light, cellular response to high light intensity, and cellular response to far red light. The DEGs of R_vs._Y were specifically enriched in cold response and processes, including GDP—mannose biosynthesis, galacturonate biosynthesis, farnesyl diphosphate biosynthesis, and abscisic acid biosynthesis (Figure 3C; Table S2).

The KEGG pathway enrichment results showed that pathways such as tropane, piperidine and pyridine alkaloid biosynthesis, sphingolipid metabolism, photosynthesis, phenylpropanoid biosynthesis, phenylalanine metabolism, galactose metabolism, flavonoid biosynthesis, and cyanoamino acid metabolism were enriched commonly in the R_vs._Y, R_vs._P, and P_vs._Y comparison groups. The DEGs of the P_vs._Y comparison group were specifically enriched in tryptophan metabolism, pentose phosphate pathway, and isoflavonoid biosynthesis. Finally, the R_vs._P DEGs were specifically enriched in ubiquinone and other terpenoid—quinone biosynthesis, riboflavin metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, fructose and mannose metabolism, flavone and flavonol biosynthesis, fatty acid elongation, carbon fixation in photosynthetic organisms, and anthocyanin biosynthesis (Figure 3D; Table S3).

3.4. Differentially Accumulated Metabolites among Yellow, Red, and Purple Wolfberry Fruits

In this study, the orthogonal partial least squares discriminant analysis (OPLS-DA) models were established and the 200 permutation cross validation tests were performed

by SMICA. As shown in Figure 4A–C, the OPLS-DA models' R2Y and Q2 values between the yellow, red, and purple groups indicated that the model had good stability, no over-fitting, and could be used to calculate the variable importance for the projection (VIP) of metabolites (Table S4; Figure 4A–C). A cluster heatmap of the differential metabolites among yellow, red, and purple wolfberry fruits was plotted and it was found that alkaloids, nucleotides, and amino acids and their derivatives, flavonoids, phenols, vitamins, organic acids, anthocyanins, and other types of compounds were differentially accumulated in yellow, red, and purple wolfberry fruits (Figure 4D). Interestingly, we found that purple wolfberries tended to accumulate more malvidin and petunidin 3,5-diglucoside, narirutin, naringin, prunin and phloridzin compared to red and yellow wolfberries; leucodelphinidin, eriodictyol, 6,8-di-C-glucoside apigenine, tricin o-saccharic acid, sakuranetin, apigenin 7-O-glucoside and C-hexosyl-tricetin O-pentoside tended to accumulate in yellow wolfberries; flavanones like naringenin, hesperidin, naringenin-7-O-glucoside tended to accumulate in red wolfberries (Table 3). Thus, these results show that flavonoids and anthocyanins are directly related to the color differences between wolfberry fruits.

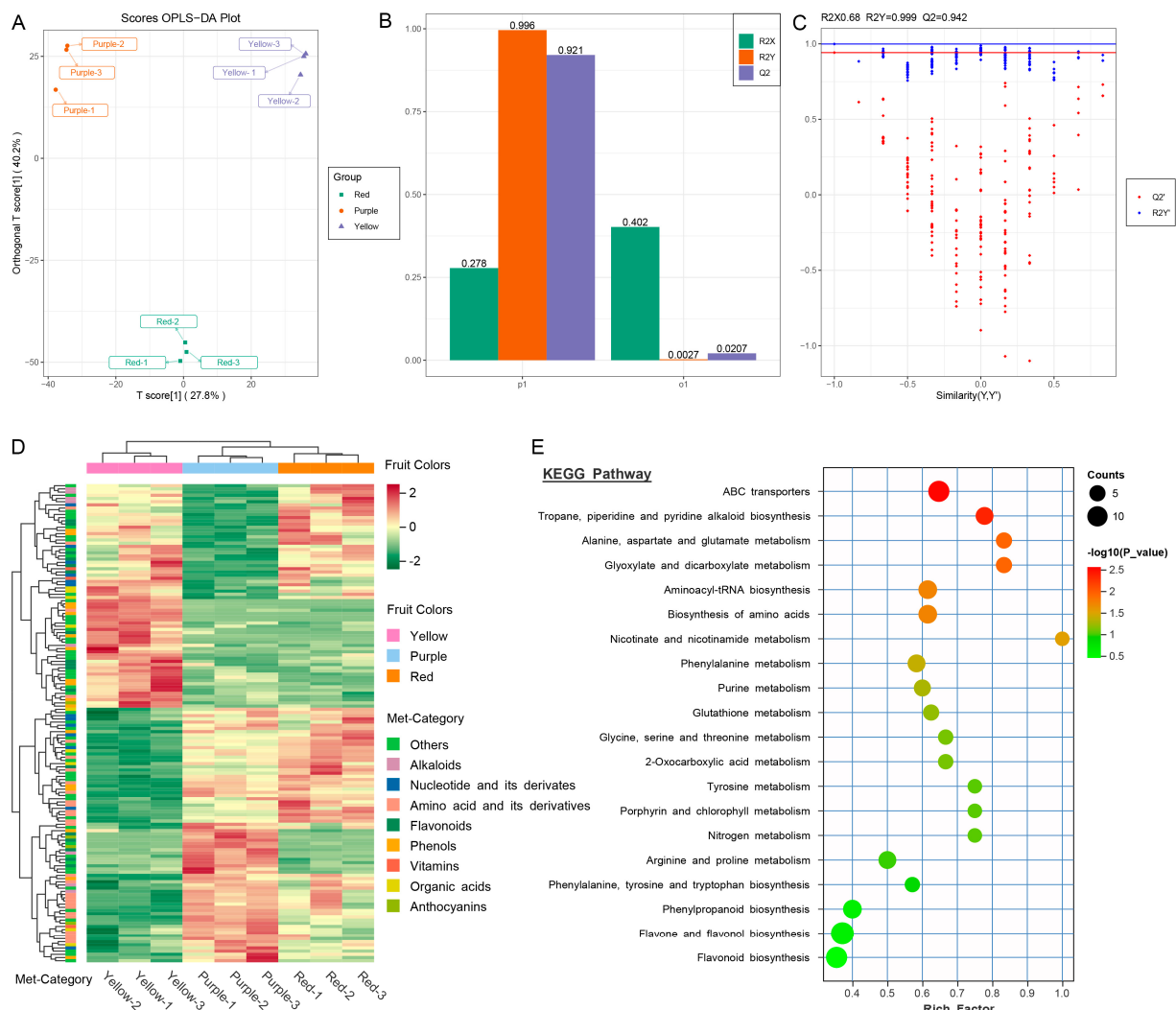


Figure 4. Statistical and functional enrichment analysis of differentially accumulated metabolites. (A) The OPLS-DA score plot. (B) The parameters of the OPLS-DA model. (C) Permutation test plot of OPLS-DA. (D) The hierarchical cluster heatmap of DAMS among yellow, red and purple fruits. (E) The bubble plot of KEGG enrichment analysis of DAMS.

Table 3. Differentially accumulated flavonoids and anthocyanin metabolites in wolfberry fruits.

Compounds	Class	Relative Content			VIP	p Value
		Red	Purple	Yellow		
Malvidin-Rut	Anthocyanins	1.54×10^4	3.48×10^5	1.18×10^4	1.68	7.17×10^{-6}
Leucodelphinidin-Hex	Anthocyanins	4.54×10^6	4.33×10^6	6.14×10^6	1.55	1.72×10^{-2}
Cyanidin-3-galactoside chloride	Anthocyanins	1.75×10^6	3.76×10^6	6.57×10^5	1.85	9.11×10^{-5}
petunidin 3,5-diglucoside	Anthocyanins	1.34×10^5	8.32×10^5	7.83×10^4	1.77	7.82×10^{-4}
Narirutin	Flavanone	2.22×10^4	1.21×10^5	3.30×10^4	1.34	1.91×10^{-4}
Naringin	Flavanone	1.18×10^4	7.95×10^5	2.17×10^4	1.50	5.86×10^{-5}
Naringenin 7-O-glucoside (Prunin)	Flavanone	3.71×10^5	5.51×10^7	4.13×10^5	1.63	3.41×10^{-6}
Naringenin	Flavanone	1.82×10^6	5.92×10^5	1.54×10^6	1.47	3.78×10^{-4}
Eriodictyol	Flavanone	3.32×10^3	1.96×10^4	1.16×10^5	1.11	1.48×10^{-3}
Hesperetin 7-rutinoside (Hesperidin)	Flavanone	4.03×10^8	2.93×10^8	3.85×10^8	1.26	3.06×10^{-2}
Naringenin-7-O-glucoside	Flavanone	6.32×10^5	2.16×10^5	4.78×10^4	1.08	4.99×10^{-4}
6,8-di-C-glucoside Apigenine	Flavone	6.77×10^3	7.26×10^3	2.26×10^4	1.38	4.03×10^{-3}
Chrysoeriol 7-O-hexoside	Flavone	6.88×10^5	4.23×10^5	7.12×10^5	1.38	4.60×10^{-2}
Apigenin 7-O-glucoside (Cosmosiin)	Flavone	7.61×10^5	1.68×10^5	7.43×10^5	1.60	5.63×10^{-4}
Tricin O-saccharic acid	Flavone	4.90×10^5	2.77×10^5	1.01×10^6	1.67	2.43×10^{-2}
sakuranetin	Flavone	1.17×10^3	1.13×10^3	3.49×10^3	1.34	2.87×10^{-2}
Apigenin 7-O-glucoside	Flavone	1.33×10^6	3.54×10^5	1.60×10^6	1.66	1.39×10^{-2}
C-hexosyl-tricetin O-pentoside	Flavonoid	6.19×10^4	6.49×10^4	2.31×10^5	1.47	9.02×10^{-3}
Phloridzin	Flavonoid	1.56×10^5	3.35×10^5	1.32×10^5	1.67	3.35×10^{-4}
Pinobanksin	Flavonol	2.81×10^6	1.02×10^6	2.44×10^6	1.48	6.13×10^{-4}

In order to understand the function of these different metabolites, KEGG pathway enrichment analysis was conducted. It was found that the DAMs among the three groups were significantly enriched in pathways such as ABC transporters, tropane, piperidine and pyridine alkaloid biosynthesis, alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, aminoacyl-tRNA biosynthesis, the biosynthesis of amino acids, nicotinate and nicotinamide metabolism, phenylalanine metabolism, purine metabolism, glutathione metabolism, and glycine, serine, and threonine metabolism. Additionally, it was found that many pathways were associated with flavonoids and anthocyanins. These include phenylalanine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, phenylpropanoid biosynthesis, and flavonoid, flavone, and flavonol biosynthesis (Figure 4E; Table S5).

3.5. Integrative Analysis of Transcriptome and Metabolome

To globally understand the regulation network of flavonoid and anthocyanins synthesis in wolfberry fruit, basing on the functional enrichment analysis results, the DEGs and DAMS which enriched in the flavonoid, flavone, and flavonol biosynthesis pathway were selected to conduct the transcriptomics and metabolomics integrative analysis (Tables S6 and S7). Aiming to discover signatory genes and metabolites involved in flavonoid and anthocyanins synthesis, data integration with O2PLS was performed on both datasets to reduce the noise and number of dominant correlations allowing for more confident biological interpretation and predictions. The O2PLS results of transcriptomics and metabolomics dataset revealed that flavonoids like hesperidin, trifolin, rutin, nictiflorin, prunin, neohesperidin, and isotrifoliin A were strongly correlated with genes such as CCG033659(F3H), CCG036272(AOMT), CCG041018(ANS), CCG028824(DFR), and CCG025288(CHS) (Figure 5A,B). The Pearson correlation analysis and correlation network suggested that prunin showed significant positive correlation with most DEGs in flavonoid biosynthesis and flavone and flavonol biosynthesis, and isotrifoliin showed significant negative correlation with these genes (Figure 5C,D).

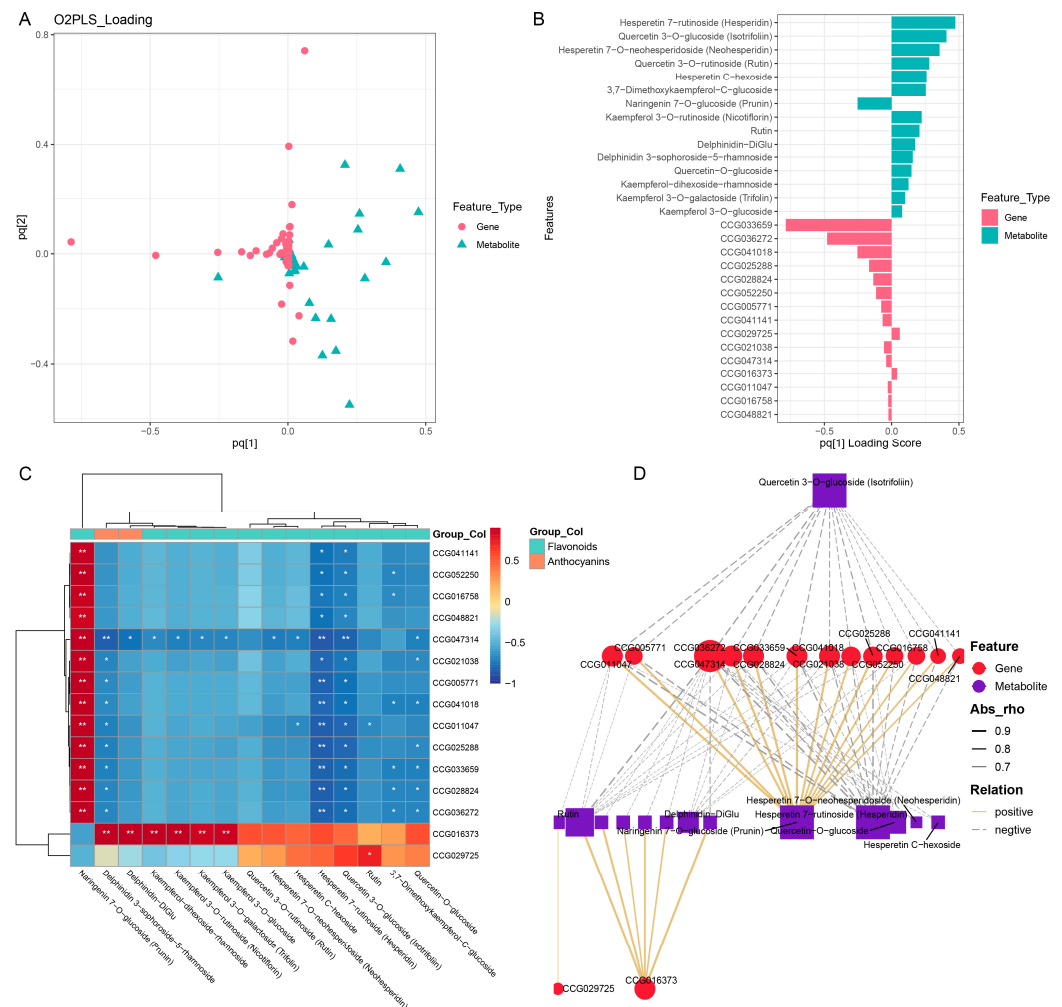


Figure 5. Integrated analysis of the DEGs and DAMs. (A) The O2PLS loading plot from the gene (p) and metabolite (q) loadings blocks of the DEGs and DAMs. Genes (circles) and metabolites (triangles) represent individual gene and metabolite loading values (B) The loading score barplot of the top 15 related metabolites and genes. (C) Hierarchical cluster heatmap of the Pearson correlation analysis of top 15 related metabolites and genes. “**” and “***” refer to Pearson correlation coefficients significance levels <0.05 and <0.01, respectively. (D) Correlation network diagram of the top 15 metabolites and genes.

4. Discussion

4.1. The Composition and Content Difference of Flavonoids Result in the Fruit Color Difference of Wolfberry

Metabolomics is an effective tool for measuring, identifying, and quantifying metabolites that have been used in various plant species [16,23]. In this study, the widely targeted metabolomic approach was adopted to investigate the metabolic changes in yellow, red, and purple wolfberry fruits. It is known that flavonoids, including flavones, isoflavones, flavanols, flavanones, and anthocyanins, play a vital role in plant tissue color formation [24–26]. The color of a fruit is essentially determined by its intrinsic substance and its content [27,28]. In our study, the metabolome results of three typical color differences of *Lycium barbarum* showed that red, yellow and purple wolfberry had significant differences in the accumulation of metabolites. Many DAMs between the three different colors are flavonoid compounds, such as flavonols and anthocyanins. And the accumulation pattern of flavonoids of wolfberries in different colors was different: anthocyanins tended to accumulate in purple wolfberry, for example, malvidin and petunidin 3,5-diglucoside; flavones tended to accumulate in yellow wolfberry, such as 6,8-di-C-glucoside apigenine,

tricin O-saccharic acid and sakuranetin; flavanones like naringenin, hesperidin, naringenin-7-O-glucoside tended to accumulate in red wolfberries. When anthocyanins, flavonoids, and flavonols are mixed according to different proportions and contents, they will show different color characteristics [29], which means that the differences in flavonoid composition and content are the material basis for the color difference of wolfberry fruits. Anthocyanins are important pigmentation pigments in plant, with a color gamut ranging from red to purple, for example, malvidin and petunidin which accumulate in purple wolfberry appear bluish-red in color [25,26,29,30]. While yellowish flavonoids, which play a key role in plant coloring [31], such as apigenine [32], sakuranetin, naringenin [33], and hesperidin accumulate in red and yellow wolfberries. In summary, we hypothesized that purple wolfberries could synthesize or accumulate more bluish anthocyanins and appear dark purple, while yellow and red wolfberries may contain more yellowish flavonoids and appear yellow and red. Therefore, *Lycium barbarum* cultivars with different colors can be obtained by controlling the biosynthesis and accumulation of anthocyanins or other flavonoids in breeding.

4.2. The Expression Differences of Flavonoid Biosynthesis Genes Reshape the Carbon Flow in Flavonoid Metabolic Pathways

In this study, KEGG pathway enrichment analysis showed that phenylalanine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, phenylpropanoid biosynthesis, and flavonoid, flavone, and flavonol biosynthesis pathways were enriched in the DAMs among yellow, red, and purple wolfberry fruits (Figure 4E). Anthocyanidin biosynthesis belongs to a branch pathway of flavonoids that starts from phenylalanine (Figure 6). A variety of enzymes participate in the biosynthesis of anthocyanins in higher plants [34]. In our study, the transcriptomics results showed that phenylpropanoid biosynthesis, phenylalanine metabolism, flavonoid biosynthesis, isoflavonoid biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, flavone and flavonol biosynthesis, and anthocyanin biosynthesis were enriched in the DEGs between yellow, red, and purple wolfberry fruits (Figure 3C,D).

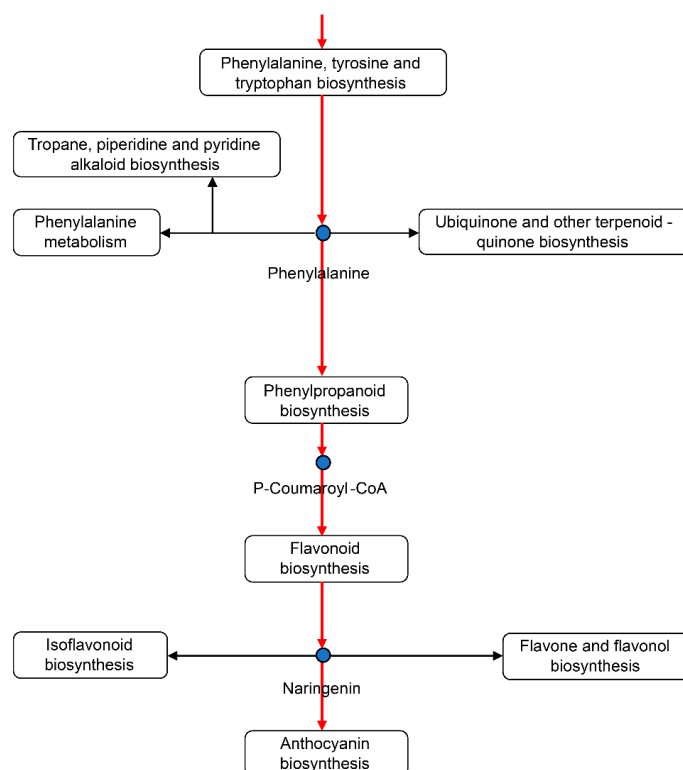


Figure 6. Schematic diagram of the anthocyanin biosynthesis pathway [9,10].

Chalcone synthase (CHS) is a key enzyme in the flavonoid biosynthesis pathway [35,36]. Previous studies revealed that CHS is a key regulatory protein for anthocyanin biosynthesis in red nectarines and peaches, and the downregulation of the CHS gene could switch the anthocyanin pathway to chlorogenic acid synthesis [37]. In this study, a total of 9 CHS genes were identified by RNA-sequencing, among which CCG011047 and CCG025288 were differentially expressed between red, yellow, and purple wolfberries, and purple wolfberry showed the highest expression level of these CHS genes. Flavonone 3-hydroxylase (F3H), dihydroflavonol-4-reductase (DFR), and anthocyanin synthase (ANS) are key enzymes in the synthesis of flavonols and anthocyanins [10]. Our study revealed that an ANS-coding gene (CCG041018), two F3H-coding genes (CCG019697 and CCG033659), and one DFR-coding genes (CCG028824) were differentially expressed between red, yellow, and purple wolfberries. Interestingly, most of these genes also expressed high levels in the purple wolfberries. Metabolomics showed that purple wolfberry synthesized and accumulated more bluish anthocyanins, and transcriptomics showed that the key enzyme genes of anthocyanins biosynthesis remained at higher expression levels in purple wolfberry than red and yellow ones, suggesting that controlling or modifying the key enzyme genes of anthocyanin synthesis could control the fruit color of wolfberry.

Besides these enzymes above, a lot of studies have shown that MYB and bHLH transcription factors also play important roles in plant coloring [23,24,38–41]. For instance, Wang et al. found a promising candidate flavonoid for regulating transcription factor LrMYB1, which can induce ectopic flavonoid accumulation in *L. ruthenicum* [42]. Lin Tang et al. found that the abnormal expression of LrAN1b, a bHLH transcription factor, results in a white fruit phenotype in *Lycium ruthenicum* [18]. In our transcriptome study, it was shown that several MYB and bHLH genes were differentially expressed in different colored wolfberry fruits and particularly highly expressed in the fruits of purple wolfberry. The transcriptomics results are consistent with the metabolomics results. Therefore, it can be speculated that MYB or bHLH transcription factors could regulate flavonoids biosynthesis related genes to control the composition of different flavonoids or anthocyanins in wolfberry fruits and result in the different fruit colors.

5. Conclusions

In this study, metabolomics and transcriptomics analysis were integrated to elucidate the regulatory mechanisms of flavonoids biosynthesis in red, yellow, and purple wolfberry. Metabolomics analysis revealed that flavonoids, phenols, vitamins, anthocyanins, and other compounds differentially accumulated among yellow and purple wolfberry fruits. And the flavonoid composition and content of wolfberries of different colors were different: the bluish anthocyanins, malvidin and petunidin tended to accumulate in purple wolfberry, while red and yellow wolfberries tended to accumulate more yellowish flavonoids. The different flavonoid accumulation patterns may result in the different fruit colors of wolfberry. Transcriptome analysis showed that numerous flavonoid-synthesis-related genes such as CHS, F3H, ANS, and DFR, and several MYB and bHLH genes were differentially expressed among wolfberry fruits of different colors: most of these genes were more highly expressed in purple wolfberries than in red and yellow wolfberries. The different expression profiles of flavonoid biosynthesis genes and MYB and bHLH genes could reshape flavonoid metabolic pathways and regulate the fruit color of wolfberry. In conclusion, the different flavonoid accumulation patterns may result in the different fruit colors of wolfberry, and the MYB or bHLH transcription factors could regulate the expression of flavonoid-biosynthesis-related genes to change the composition of flavonoids or anthocyanins in wolfberry fruits and result in various fruit colors. These findings provide new insights into the underlying molecular mechanisms of the fruit color differences in wolfberry and provide new ideas for molecular breeding of wolfberry.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy13071926/s1>, Table S1: All differentially expressed genes between red, purple, and yellow wolfberry fruits; Table S2: GO functional enrichment results of differentially expressed genes between red, purple, and yellow wolfberry fruits; Table S3: KEGG functional enrichment results of differentially expressed genes between red, purple, and yellow wolfberry fruits. “*”, “**”, “***” and “****” refer to significance levels <0.05, <0.01, <0.005 and <0.001, respectively; Table S4: All different metabolites between red, yellow, and purple wolfberry fruits; Table S5: KEGG functional enrichment results of different metabolites between red, purple, and yellow wolfberry fruits; Table S6: DEGs related to flavonoids biosynthesis in wolfberry fruits; Table S7: Different metabolites involved in flavonoids biosynthesis between wolfberry fruits.

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Conflicts of Interest: The authors declare no conflict of interest.

References

- Wang, H.; Li, J.; Tao, W.; Zhang, X.; Gao, X.; Yong, J.; Zhao, J.; Zhang, L.; Li, Y.; Duan, J.A. *Lycium ruthenicum* studies: Molecular biology, Phytochemistry and pharmacology. *Food Chem.* **2018**, *240*, 759–766. [\[CrossRef\]](#) [\[PubMed\]](#)
- Yao, R.; Heinrich, M.; Zou, Y.; Reich, E.; Zhang, X.; Chen, Y.; Weckerle, C.S. Quality Variation of Goji (*Fruits of Lycium* spp.) in China: A Comparative Morphological and Metabolomic Analysis. *Front. Pharmacol.* **2018**, *9*, 151. [\[CrossRef\]](#) [\[PubMed\]](#)
- Xiao, Z.; Deng, Q.; Zhou, W.; Zhang, Y. Immune activities of polysaccharides isolated from *Lycium barbarum* L. What do we know so far? *Pharmacol. Ther.* **2022**, *229*, 107921. [\[CrossRef\]](#)
- Liu, Y.; Tikunov, Y.; Schouten, R.E.; Marcelis, L.F.M.; Visser, R.G.F.; Bovy, A. Anthocyanin Biosynthesis and Degradation Mechanisms in Solanaceous Vegetables: A Review. *Front. Chem.* **2018**, *6*, 52. [\[CrossRef\]](#)
- Meng, Y.; Zhang, H.; Fan, Y.; Yan, L. Anthocyanins accumulation analysis of correlated genes by metabolome and transcriptome in green and purple peppers (*Capsicum annuum*). *BMC Plant Biol.* **2022**, *22*, 358. [\[CrossRef\]](#) [\[PubMed\]](#)
- Jiang, T.; Zhang, M.; Wen, C.; Xie, X.; Tian, W.; Wen, S.; Lu, R.; Liu, L. Integrated metabolomic and transcriptomic analysis of the anthocyanin regulatory networks in *Salvia miltiorrhiza* Bge. flowers. *BMC Plant Biol.* **2020**, *20*, 349. [\[CrossRef\]](#)
- Tanaka, Y.; Brugliera, F.; Chandler, S. Recent progress of flower colour modification by biotechnology. *Int. J. Mol. Sci.* **2009**, *10*, 5350–5369. [\[CrossRef\]](#)
- Peng, J.; Dong, X.; Xue, C.; Liu, Z.; Cao, F. Exploring the Molecular Mechanism of Blue Flower Color Formation in *Hydrangea macrophylla* cv. “Forever Summer”. *Front. Plant Sci.* **2021**, *12*, 585665. [\[CrossRef\]](#) [\[PubMed\]](#)
- Tanaka, Y.; Sasaki, N.; Ohmiya, A. Biosynthesis of plant pigments: Anthocyanins, betalains and carotenoids. *Plant J.* **2008**, *54*, 733–749. [\[CrossRef\]](#)
- Liu, W.; Feng, Y.; Yu, S.; Fan, Z.; Li, X.; Li, J.; Yin, H. The Flavonoid Biosynthesis Network in Plants. *Int. J. Mol. Sci.* **2021**, *22*, 12824. [\[CrossRef\]](#)
- Shen, N.; Wang, T.; Gan, Q.; Liu, S.; Wang, L.; Jin, B. Plant flavonoids: Classification, distribution, biosynthesis, and antioxidant activity. *Food Chem.* **2022**, *383*, 132531. [\[CrossRef\]](#)
- Hirasawa, T.; Saito, M.; Yoshikawa, K.; Furusawa, C.; Shmizu, H. Integrated Analysis of the Transcriptome and Metabolome of *Corynebacterium glutamicum* during Penicillin-Induced Glutamic Acid Production. *Biotechnol. J.* **2018**, *13*, e1700612. [\[CrossRef\]](#)
- Mesnage, R.; Biserni, M.; Balu, S.; Frainay, C.; Poupin, N.; Jourdan, F.; Wozniak, E.; Xenakis, T.; Mein, C.A.; Antoniou, M.N. Integrated transcriptomics and metabolomics reveal signatures of lipid metabolism dysregulation in HepaRG liver cells exposed to PCB 126. *Arch. Toxicol.* **2018**, *92*, 2533–2547. [\[CrossRef\]](#) [\[PubMed\]](#)
- Li, N.; Wang, J.; Wang, B.; Huang, S.; Hu, J.; Yang, T.; Asmutola, P.; Lan, H.; Qinghui, Y. Identification of the Carbohydrate and Organic Acid Metabolism Genes Responsible for Brix in Tomato Fruit by Transcriptome and Metabolome Analysis. *Front. Genet.* **2021**, *12*, 714942. [\[CrossRef\]](#) [\[PubMed\]](#)

15. Gong, C.; Zhu, H.; Lu, X.; Yang, D.; Zhao, S.; Umer, M.J.; He, N.; Yuan, P.; Anees, M.; Diao, W.; et al. An integrated transcriptome and metabolome approach reveals the accumulation of taste-related metabolites and gene regulatory networks during watermelon fruit development. *Planta* **2021**, *254*, 35. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Wang, Y.; Zhang, X.; Yang, S.; Yuan, Y. Lignin Involvement in Programmed Changes in Peach-Fruit Texture Indicated by Metabolite and Transcriptome Analyses. *J. Agric. Food Chem.* **2018**, *66*, 12627–12640. [\[CrossRef\]](#)
17. Wang, Z.; Song, Q.; Shuai, L.; Htun, R.; Malviya, M.K.; Li, Y.; Liang, Q.; Zhang, G.; Zhang, M.; Zhou, F. Metabolic and proteomic analysis of nitrogen metabolism mechanisms involved in the sugarcane-Fusarium verticillioides interaction. *J. Plant Physiol.* **2020**, *251*, 153207. [\[CrossRef\]](#)
18. Li, T.; Fan, Y.; Qin, H.; Dai, G.; Li, G.; Li, Y.; Wang, J.; Yin, Y.; Chen, F.; Qin, X.; et al. Transcriptome and Flavonoids Metabolomic Analysis Identifies Regulatory Networks and Hub Genes in Black and White Fruits of *Lycium ruthenicum* Murray. *Front. Plant Sci.* **2020**, *11*, 1256. [\[CrossRef\]](#)
19. Sun, X.; Zheng, Y.; Tian, L.; Miao, Y.; Zeng, T.; Jiang, Y.; Pei, J.; Ahmad, B.; Huang, L. Metabolome profiling and molecular docking analysis revealed the metabolic differences and potential pharmacological mechanisms of the inflorescence and succulent stem of *Cistanche deserticola*. *RSC Adv.* **2021**, *11*, 27226–27245. [\[CrossRef\]](#) [\[PubMed\]](#)
20. Zhou, J.; Hou, D.; Zou, W.; Wang, J.; Luo, R.; Wang, M.; Yu, H. Comparison of Widely Targeted Metabolomics and Untargeted Metabolomics of Wild *Ophiocordyceps sinensis*. *Molecules* **2022**, *27*, 3645. [\[CrossRef\]](#)
21. Cao, Y.L.; Li, Y.L.; Fan, Y.F.; Li, Z.; Yoshida, K.; Wang, J.Y.; Ma, X.K.; Wang, N.; Mitsuda, N.; Kotake, T.; et al. Wolfberry genomes and the evolution of Lycium (Solanaceae). *Commun. Biol.* **2021**, *4*, 671. [\[CrossRef\]](#)
22. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [\[CrossRef\]](#)
23. Xiao, L.; Cao, S.; Shang, X.; Xie, X.; Zeng, W.; Lu, L.; Kong, Q.; Yan, H. Metabolomic and transcriptomic profiling reveals distinct nutritional properties of cassavas with different flesh colors. *Food Chem.* **2021**, *2*, 100016. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Xu, W.; Dubos, C.; Lepiniec, L. Transcriptional control of flavonoid biosynthesis by MYB-bHLH-WDR complexes. *Trends Plant Sci.* **2015**, *20*, 176–185. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Gawande, V.; Vidya, V.; Kalikar, S.V. Flavonoids: An Overview. *J. Pharm. Res.* **2012**, *5*, 5099–5101. [\[CrossRef\]](#)
26. Alappat, B.; Alappat, J. Anthocyanin Pigments: Beyond Aesthetics. *Molecules* **2020**, *25*, 5500. [\[CrossRef\]](#)
27. Ferreira, V.; Pinto-Carnide, O.; Arroyo-García, R.; Castro, I. Berry color variation in grapevine as a source of diversity. *Plant Physiol. Biochem.* **2018**, *132*, 696–707. [\[CrossRef\]](#)
28. Ilahy, R.; Tlili, I.; Siddiqui, M.W.; Hdider, C.; Lenucci, M.S. Inside and Beyond Color: Comparative Overview of Functional Quality of Tomato and Watermelon Fruits. *Front. Plant Sci.* **2019**, *10*, 769. [\[CrossRef\]](#)
29. Cui, B.; Hu, Z.; Zhang, Y.; Hu, J.; Yin, W.; Feng, Y.; Xie, Q.; Chen, G. Anthocyanins and flavonols are responsible for purple color of *Lablab purpureus* (L.) sweet pods. *Plant Physiol. Biochem.* **2016**, *103*, 183–190. [\[CrossRef\]](#)
30. Chamizo-González, F.; Estévez, I.G.; Gordillo, B.; Manjón, E.; Escribano-Bailón, M.T.; Heredia, F.J.; González-Miret, M.L. First insights into the binding mechanism and colour effect of the interaction of grape seed 11S globulin with malvidin 3-O-glucoside by fluorescence spectroscopy, differential colorimetry and molecular modelling. *Food Chem.* **2023**, *413*, 135591. [\[CrossRef\]](#)
31. Taniguchi, M.; LaRocca, C.A.; Bernat, J.D.; Lindsey, J.S. Digital Database of Absorption Spectra of Diverse Flavonoids Enables Structural Comparisons and Quantitative Evaluations. *J. Nat. Prod.* **2023**, *86*, 1087–1119. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Han, A.R.; Nam, B.; Kim, B.R.; Lee, K.C.; Song, B.S.; Kim, S.H.; Kim, J.B.; Jin, C.H. Phytochemical Composition and Antioxidant Activities of Two Different Color Chrysanthemum Flower Teas. *Molecules* **2019**, *24*, 329. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Zhang, Y.; Zhao, G.; Li, Y.; Zhang, J.; Shi, M.; Muhammad, T.; Liang, Y. Transcriptome Profiling of Tomato Uncovers an Involvement of Cytochrome P450s and Peroxidases in Stigma Color Formation. *Front. Plant Sci.* **2017**, *8*, 897. [\[CrossRef\]](#)
34. Zhang, Q.; Su, L.J.; Chen, J.W.; Zeng, X.Q.; Peng, S.L. The antioxidative role of anthocyanins in Arabidopsis under high-irradiance. *Biol. Plant.* **2012**, *56*, 97–104. [\[CrossRef\]](#)
35. Pandith, S.A.; Ramazan, S.; Khan, M.I.; Reshi, Z.A.; Shah, M.A. Chalcone synthases (CHSs): The symbolic type III polyketide synthases. *Planta* **2019**, *251*, 15. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Ferrer, J.L.; Austin, M.B.; Stewart, C., Jr.; Noel, J.P. Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiol. Biochem.* **2008**, *46*, 356–370. [\[CrossRef\]](#)
37. Rahim, M.A.; Busatto, N.; Trainotti, L. Regulation of anthocyanin biosynthesis in peach fruits. *Planta* **2014**, *240*, 913–929. [\[CrossRef\]](#)
38. Chen, Z.; Yu, L.; Liu, W.; Zhang, J.; Wang, N.; Chen, X. Research progress of fruit color development in apple (*Malus domestica* Borkh.). *Plant Physiol. Biochem.* **2021**, *162*, 267–279. [\[CrossRef\]](#)
39. Zhao, M.; Li, J.; Zhu, L.; Chang, P.; Li, L.; Zhang, L. Identification and Characterization of MYB-bHLH-WD40 Regulatory Complex Members Controlling Anthocyanidin Biosynthesis in Blueberry Fruits Development. *Genes* **2019**, *10*, 496. [\[CrossRef\]](#)
40. Cui, D.; Zhao, S.; Xu, H.; Allan, A.C.; Zhang, X.; Fan, L.; Chen, L.; Su, J.; Shu, Q.; Li, K. The interaction of MYB, bHLH and WD40 transcription factors in red pear (*Pyrus pyrifolia*) peel. *Plant Mol. Biol.* **2021**, *106*, 407–417. [\[CrossRef\]](#)

41. Tingrui, M.A.; Zhang, J.; Liang, H. Plant Anthocyanin Synthesis and Gene Regulation. *Agric. Sci. Technol.* **2012**, *9*, 454–458.
42. Wang, C.; Dong, Y.; Zhu, L.; Wang, L.; Yan, L.; Wang, M.; Zhu, Q.; Nan, X.; Li, Y.; Li, J. Comparative transcriptome analysis of two contrasting wolfberry genotypes during fruit development and ripening and characterization of the LrMYB1 transcription factor that regulates flavonoid biosynthesis. *BMC Genom.* **2020**, *21*, 295. [[CrossRef](#)] [[PubMed](#)]

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