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Metabolome and transcriptome sequencing analysis reveals anthocyanins in the red flowers of black locust (*Robinia pseudoacacia* L.)

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Abstract

Black locust (*Robinia pseudoacacia* L.) flowers display a white or red color. The white flowers are a popular food in Chinese markets, while the red flowers have high ornamental value. In this study, combined analysis of the transcriptome and flavonoid metabolome was performed to investigate the key genes and metabolites involved in flower pigmentation in the red and white flowers of black locust. A total of 308 flavonoid metabolites were identified, and five anthocyanins were significantly higher in the red flowers compared to the white flowers. Transcriptome sequencing yielded about 66 Gb data, from which 53,992 unigenes were assembled. Compared with the white flowers, 1,394 unigenes were up-regulated and 1,201 unigenes were down-regulated in the red flowers. Three anthocyanin structural genes, *F3'H*, *ANS,* and *3GT*, were significantly up-regulated in the red flowers, representing the key genes for anthocyanin accumulation in the red flowers. Nine MYB and four bHLH genes were up-regulated in the red flowers, representing the candidate genes regulating the anthocyanin pathway. These results provide a theoretical basis for the development of black locust flowers as a food and also provide a foundation for the study of anthocyanin regulation in black locust.

Keywords: black locust; *Robinia pseudoacacia* L.; red flower; flavonoids; anthocyanins; metabolome; transcriptome.

Practical Application: Red flower of black locust is a food material rich in anthocyanins.

1 Introduction

Black locust (*Robinia pseudoacacia* L.) is a deciduous tree that is 10-25 m in height and is characterized by rapid growth. It is an important afforestation tree species that is widely planted throughout the world from temperate to subtropical areas (Rédei et al., 2008). Black locust flowers are milky white and rich in flavonoids, rendering them a healthy food that is very popular in Chinese markets (Wang et al., 2006; Wang, 2019). Robinia pseudoacacia f. decaisn*eana* (Carr.) Voss is a cultivar of black locust that possesses red flowers. It has high ornamental value and is often used for landscaping. At present, the molecular mechanism of the flower color difference etween the red and white flowers of black locust is unclear.

In most plants, anthocyanins are the main pigments dictating flower color. They are also an important visual signal for attracting insects for pollination (Davies et al., 2012). The anthocyanin pathway has been well characterized in model plants. Anthocyanin structural genes can be divided into early biosynthesis genes (EBGs), including chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase (F3H), and late biosynthesis genes (LBGs), including flavonoid-3'-hydroxylase (F3'H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) (Hichri et al., 2011). Anthocyanins are transferred stored in the vacuoles where they exhibit color (Wang et al., 2014b). Transcription factors from the MYB, bHLH, and WD40 gene families usually control the activity of the anthocyanin pathway (Zhao et al., 2013; Zhang et al., 2019). In horticultural plants, the expression of MYB genes plays an important role in flower and fruit coloration (Naing & Kim, 2018), such as LhMYB6, LhMYB12, and MYB12-Lat in Asiatic lily, and MdMYBA, MdMYB10, and MdMYB110a in apple (Takos et al., 2006; Chagné et al., 2013; Espley et al., 2007).

Black locust flowers, as forest by-products, have potential market value. Researchers have analyzed the functions and components of extracts from various flowers; for instance, Kim et al. (2011) reported that flower extracts have antioxidant activity and alleviate DNA damage, and Ma et al. (2021) analyzed red and white flowers and found 11 flavonoid glycosides. However, genes related to black locust flower coloration have not been reported. In this study, the red and white flowers of black locust were subjected to combined metabolome and transcriptome analysis, and the differences in flavonoid components were explored and the key genes controlling flower color were screened. The results will provide a reference for the development of black locust flowers as a food as well as for the cultivation of anthocyanin-rich varieties.

2 Materials and methods

2.1 Flavonoid metabolite detection

Black locust plants were grown in Longmen Mountain (Luoyang, China), and white flowers (WF) and red flowers (RF) in

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the semi-open stage were sampled on April 12, 2021. Flavonoids were extracted and analyzed by MetWare Biotechnology Co. Ltd. (Wuhan, China). The flavonoids were detected using an ultra-high-performance electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) system. The mobile phase A was ultrapure water with 0.1% acetic acid, and mobile phase B was acetonitrile with 0.1% acetic acid. The gradient program was from 5% to 95% B for 0-9.0 min, maintained at 95% B for 1 min; and from 95% to 5% B for 11.0-12.1 min, maintained at 5% B until 14.0 min. The effluent was scanned using an ESI-triple quadrupole-linear ion trap-MS/MS system (Applied Biosystems 4500 Q TRAP) (Chen et al., 2013).

2.2 Metabolite data analysis

All metabolites were annotated using the MetWare database and quantified using multiple reaction monitoring. The different flavonoids between WF and RF were analyzed using partial least squares-discriminant analysis (PLS-DA). The thresholds of variable importance in the projection (VIP) ≥ 1 and absolute log2FC (fold-change) ≥ 1 were used to determine significantly different flavonoids.

2.3 Transcriptome sequencing

Sequencing libraries were generated by Biomarker Technologies Co. Ltd (Beijing, China) and sequenced with an Illumina NovaSeq 6000 platform. Reads containing poly-N or adapters as well as reads that were low quality were discarded. Trinity software was used to assemble high-quality reads (Grabherr et al., 2011). The transcriptome data have been deposited in the Genome Sequence Archive (GSA) under accession number PRJCA008127 (http://bigd.big.ac.cn/gsa). Gene function was annotated by aligning against the Swiss-Prot, Pfam, Kyoto Encyclopedia of Genes and Genomes (KEGG), Eukaryotic Orthologous Groups (KOG), and Gene Ontology (GO) databases.

2.4 Differential expression analysis

Gene expression levels were estimated by RSEM (Li & Dewey, 2011), and differentially expressed genes were screened using the DESeq R package with the thresholds of q-value < 0.05 and | log2(foldchange) | > 1.

2.5 Quantitative real-time PCR analysis

The first-strand cDNA was synthesized using the Prime Script™ RT reagent Kit with gDNA Eraser (TaKaRa, China). Transcriptional levels of flavonoid structural genes were detected using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, China) on a CFX96TM Real-Time System (Bio-Rad, USA). The quantitative real-time PCR (qRT-PCR) reaction was performed with TB Green Premix Ex Taq II. The amplification program was as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s. *GADPH* was used as an internal control (Wang et al., 2014a). Primers used for qRT-PCR are shown in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression (Livak & Schmittgen, 2001).

3 Results

3.1 UPLC-MS/MS-based quantitative metabolomic analysis of black locust flowers

In order to elucidate the flavonoid components of black locust flowers, the flavonoid metabolites of RF and WF were analyzed. A total of 308 flavonoid metabolites were identified, including 20 anthocyanins, 12 chalcones, 10 flavanols, 19 flavanones, nine flavanonols, 64 flavones, 18 flavonoid carbonosides, 97 flavonols, 40 isoflavones, nine proanthocyanidins, and 10 tannins (Table S1).

A hierarchical clustering heatmap was constructed using the quantitative metabolite data. As shown in Figure 1, there was a clear separation between the red and white flower samples. Principal component analysis also showed an obvious separation between the WF and RF samples (Figure S1). These results indicated that the flavonoid profiles differed between RF and WF.

3.2 Flavonoid metabolome profiling of RF and WF samples

Differential compounds between WF and RF were screened using PLS-DA models, and a total of 175 differential metabolites were identified. Compared with WF, 123 compounds were up-regulated and 52 compounds were down-regulated in RF (Figure 2). Five anthocyanins were significantly up-regulated in RF, including malvidin-3-*O*-arabinoside, delphinidin-3-*O*-galactoside, rosinidin-3-*O*-glucoside, cyanidin-3-*O*-(2"-*O*-glucosyl)glucoside, and delphinidin-3,5-di-*O*-glucoside, and these represent the main pigments of the red flowers.

3.3 De novo assembly

The transcriptomes of the black locust flowers were sequenced. About 11.3 Gb, 11.5 Gb, and 10.9 Gb bases of clean data were obtained from the three RF samples, and 11.2 Gb, 11.2 Gb, and 10.4 Gb bases of clean data were obtained from the three WF samples. After *de novo* assembly, 53,992 unigenes were obtained with an N50 of 1817 nt. There were 19,633 unigenes with a

Table 1. Primers used for quantitative real-time PCR.

Gene	Forward primer	Reverse sequence
GAPDH	TCAACAATGCCAAACCTG	GTGTCAACGAGCACGAAT
F3'H	TCTCAGTGGTAGAAACGCCA	AATCCCCATCCTTGTCCCAG
ANS	ATCAACCGCCTCAAGAAAGC	GACCACTTGCATTGTTGGCT
3GT	TCTAGTGCAGGAAGAGGGGA	ATCATGTGCTGCTCTCCCTT
FNSII	TTCTGGAAGGAGAGGTTGCC	GTCAATCCTGGCCGTTCATC
ANR	GCATTGCTACGCACTGTATG	GTAACCAGTCCCATCATCCT

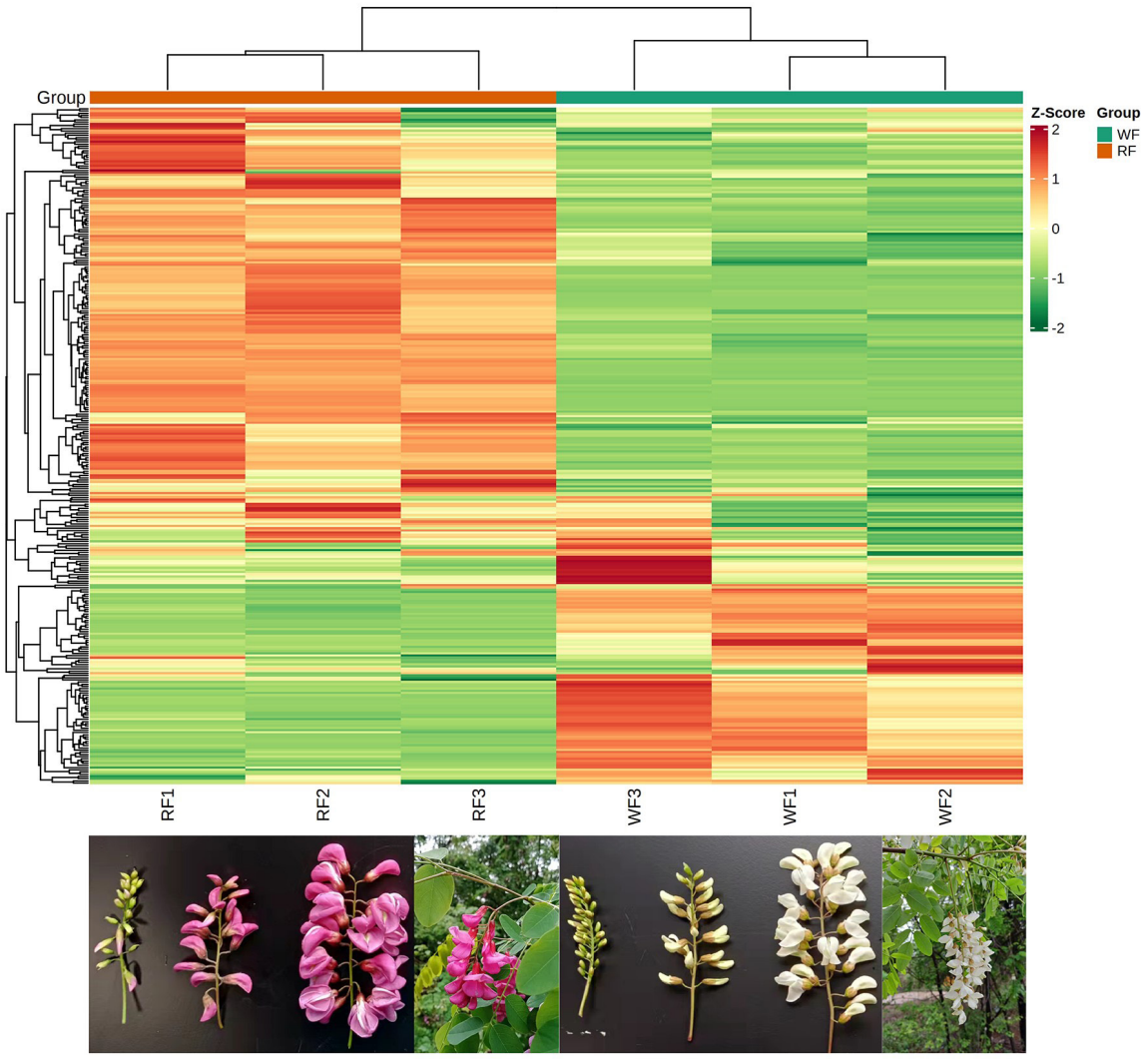

Figure 1. Hierarchical clustering heatmap of flavonoid metabolites in WF and RF.

length of 300-500 bp, accounting for 36.6% of unigenes; 15,127 unigenes with a length of 500-1,000 bp, accounting for 28.02% of unigenes; 10,382 unigenes with a length of 1,000-2,000 bp, accounting for 19.23% of unigenes; and 8,850 unigenes with a length > 2,000 bp, accounting 16.39% of unigenes. Among the unigenes, 29,132 unigenes could be annotated to nine public databases.

3.4 Analysis of differently expressed genes (DEGs) between WF and RF

Through comparative analysis of the transcriptome between WF and RF, a total of 2,595 DEGs were obtained, of which 2,264 were annotated into public databases. Compared with WF, 1,394 unigenes were up-regulated and 1,201 unigenes were down-regulated in RF (Figure 3). In terms of KEGG annotation, 1,523 unigenes were annotated to 128 pathways. Twenty-eight pathways were enriched, including three pathways related to flower color, namely "isoflavonoid biosynthesis (Ko00943)", "flavonoid biosynthesis (Ko00941)", and "flavone and flavonol biosynthesis (Ko00944)".

3.5 Identification of DEGs involved in flower color

A total of five DEGs related to flower color were identified (Table 2). Among them, three anthocyanin structural genes, including *F3'H* (c76211.graph_c0), *ANS* (c70083.graph_c0), and *3GT* (c80641.graph_c0), were up-regulated in RF. Two genes, *FNSII* (c87553.graph_c0) and *ANR* (c69641.graph_c0), involved in flavone and flavanone biosynthesis, were down-regulated in RF.

3.6 Transcriptome profiles of the transcription factors

A total of 51 transcription factors belonging to 14 families were differentially expressed in RF and WF, of which 29 were up-regulated and 22 were down-regulated in RF. MYB and bHLH play critical roles in the regulation of the anthocyanin pathway, and among the DEGs, nine MYB genes and four bHLH genes

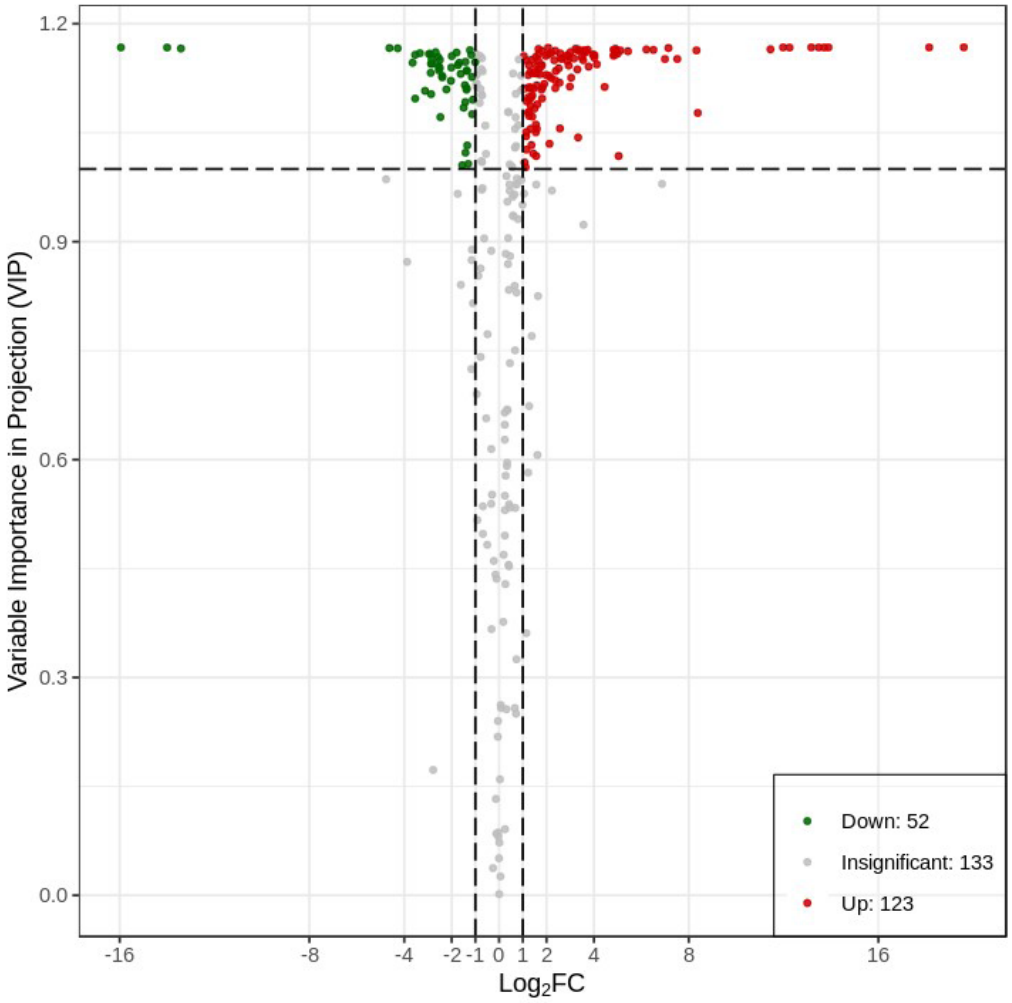

Figure 2. Volcano plot of differentially accumulated metabolites between WF and RF.

were up-regulated, and five MYB genes and four bHLH genes were down-regulated in RF (Table 3).

3.7 Quantitative RT-PCR analysis of flavonoid structural genes

The transcription levels of the five flavonoid structural genes were verified using qRT-PCR (Figure 4). The results showed that *F3'H*, *ANS*, and *3GT* were up-regulated, while *FNSII* and *ANR* were down-regulated in RF, which was consistent with the transcriptome data.

Data represent the means ± SD of three independent biological replicates. Bars represent the standard errors of three biological replicates.

4 Discussion

Flavonoids have positive health impacts, such as by decreasing the risk of cardiovascular diseases, inhibiting the development and progress of different types of cancers, and reducing gut inflammation (Kopustinskiene et al., 2020; Pei et al., 2020; Maleki et al., 2019). Therefore, foods rich in flavonoids have become increasingly popular. Analysis of the flavonoid metabolome of the white and red flowers of black locust identified 308 flavonoids, of which 175 differed between WF and RF, with 123 up-regulated and 52 down-regulated in RF. These results showed that there were great differences in the flavonoid composition between the red and white flowers. This study preliminarily identified the flavonoid profiles in WF and RF, providing a foundation for the development of black locust flowers as a flavonoid-rich food or drug.

Anthocyanins are flavonoids and are the primary pigments in most flowers. In tree peony, *Rhododendron* species, and other ornamental flowers, differences in anthocyanin contents are the key factors influencing the richness and diversity of flower colors in different varieties (Zhang et al., 2020; Du et al., 2018). Of the differential metabolites between RF and WF, five anthocyanins increased significantly, including malvidin-3-*O*-arabinoside, delphinidin-3-*O*-galactoside, rosinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, and delphinidin-3,5-di-*O*-glucoside. The results showed that the accumulation of these five types of pigments was the main contributor to the red coloration of RF.

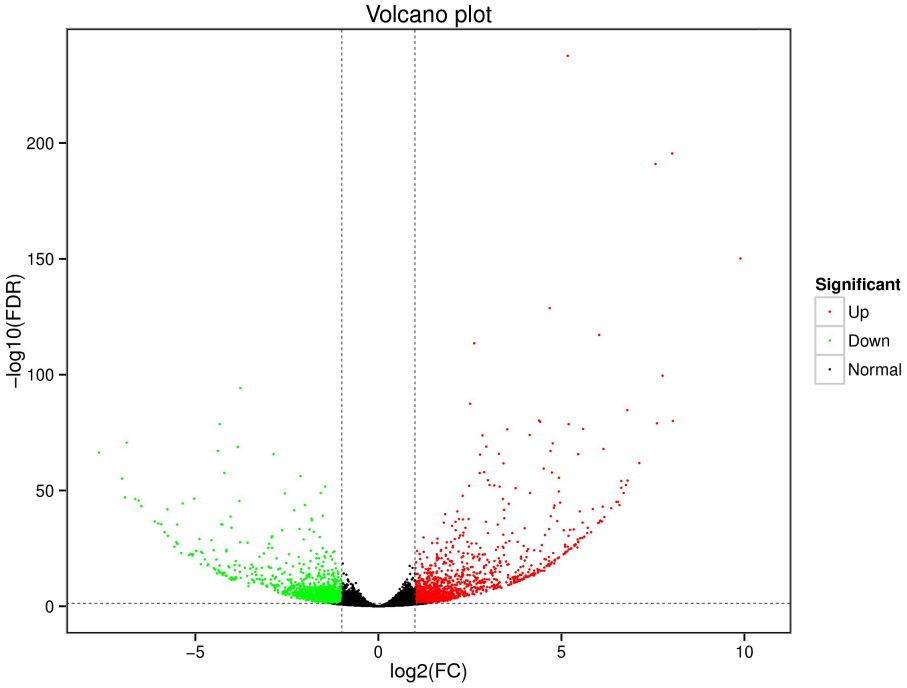

Figure 3. Analysis of differentially expressed genes in WF and RF.

Table 2. Transcript profiles of DEGs involved in the flavonoid pathway in WF and RF.

Gene name	Gene ID	WF_FPKM	RF_FPKM	FDR	log2FC	Regulated
F3'H	c76211.graph_c0	12.55333	115.2167	2.81E-32	2.9937	up
ANS	c70083.graph_c0	122.97	1036.817	1.39E-58	2.891557	up
3GT	c80641.graph_c0	135.9033	321.7067	0.003921	1.010333	up
FNSII	c87553.graph_c0	8.266667	1.31	7.29E-09	−2.42646	down
ANR	c69641.graph_c0	3.183333	0.05	2.76E-18	−4.33675	down

Table 3. Differentially expressed MYB and bHLH genes in RF and WF.

Gene family	Gene ID	WF_FPKM	RF_FPKM	FDR	log2FC	Regulated
MYB	c71325.graph_c0	3.21	7.07	1.08E-05	1.01	up
	c72408.graph_c0	0.34	3.63	1.96E-12	2.90	up
	c72163.graph_c0	0.35	2.54	3.39E-05	2.15	up
	c75524.graph_c0	1.09	4.39	6.09E-04	1.56	up
	c72849.graph_c0	1.66	4.44	1.42E-05	1.18	up
	c75341.graph_c0	0.74	3.19	3.68E-07	1.83	up
	c88685.graph_c1	30.31	116.99	2.07E-07	1.98	up
	c89531.graph_c0	0.11	7.55	1.33E-08	3.36	up
	c74223.graph_c0	1.74	8.43	7.21E-11	2.16	up
	c84655.graph_c0	5.57	2.63	1.09E-04	−1.13	down
	c72549.graph_c0	5.36	2.72	3.26E-03	−1.01	down
	c85131.graph_c2	5.35	1.16	1.40E-08	−2.33	down
	c82305.graph_c1	107.78	39.99	1.08E-05	−1.35	down
	c81473.graph_c2	29.79	11.15	7.62E-11	−1.38	down
bHLH	c74899.graph_c0	0.88	3.54	2.39E-05	1.69	up
	c88771.graph_c1	6.59	12.47	1.98E-10	1.25	up
	c77816.graph_c0	1.29	3.94	2.29E-04	1.54	up
	c86677.graph_c3	3.36	7.90	3.91E-03	1.22	up
	c78679.graph_c0	5.60	2.15	9.04E-04	−1.40	down
	c78347.graph_c0	23.76	11.03	8.87E-09	−1.18	down
	c83896.graph_c0	13.40	7.74	7.32E-08	−1.30	down
	c76319.graph_c0	22.92	3.15	1.39E-33	−2.63	down

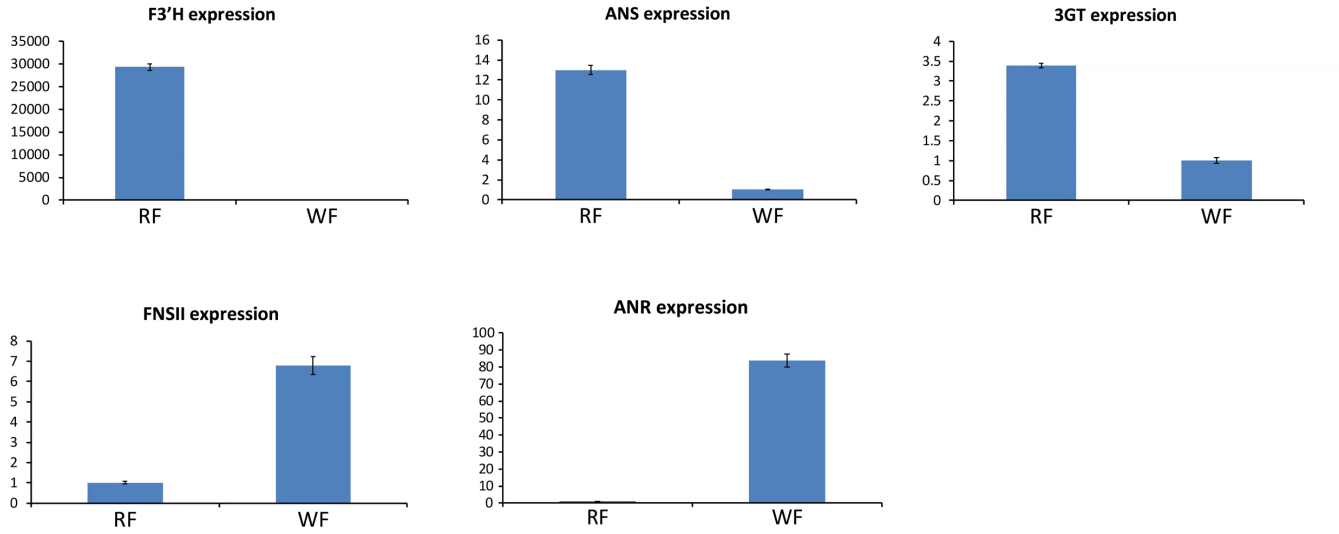

Figure 4. Expression analysis of five flavonoid structural genes by qRT-PCR. Data represent the means ± SD of three independent biological replicates. Bars represent the standard errors of three biological replicates.

Studies on the functional genes of black locust are limited, and no flavonoid-related genes have been reported thus far. With the development of high-throughput sequencing technology in recent years, transcriptome and genome sequencing technologies have also been widely applied in forest research (Yao et al., 2020; Cao et al., 2021). We sequenced the transcriptomes of WF and RF, from which we obtained 66 Gb data and assembled 53,992 unigenes. This is the first report on the transcriptome of black locust flower, providing a foundation for the study of functional genes in this species.

Through DEG analysis, it was found that the transcription levels of *F3'H*, *ANS,* and *3GT* were significantly up-regulated in RF. These three genes are key anthocyanin structural genes, and mutations in each of them can hinder anthocyanin production (Kim et al., 2005; Morita et al., 2015; Nakatsuka et al., 2005). Therefore, compared with WF, the increased transcription of the *F3'H*, *ANS,* and *3GT* genes constitutes the key contributor to anthocyanin accumulation in RF.

The MYB gene is the key regulatory gene of the anthocyanin pathway. Some MYBs mainly regulate LBGs, such as *PavMYBA* and *PavMYB10,* which primarily regulate *DFR*, *ANS,* and *UFGT* in cherry (Lin-Wang et al., 2010; Shen et al., 2014); *MdMYBA* and *MdMYB1,* which regulate *DFR*, *ANS,* and *3GT* in apple (Espley et al., 2007); and *RsMYB1,* which regulates *DFR* and *ANS* in radish (Park et al., 2011). In this study, nine MYBs were up-regulated in RF, and these genes represent the candidate genes regulating the transcription of *F3'H*, *ANS,* and *3GT* in the red flowers of black locust.

5 Conclusion

A total of 308 flavonoid metabolites were identified in the white and red flowers of black locust, and five anthocyanins were found to be the main contributors to red flower coloration. Compared with the white flowers, three anthocyanin structural genes, namely *F3'H*, *ANS,* and *3GT,* were up-regulated in the red flowers, leading to the accumulation of anthocyanins.

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References

Cao, Y., Sun, G., Zhai, X., Xu, P., Ma, L., Deng, M., Zhao, Z., Yang, H., Dong, Y., Shang, Z., Lv, Y., Yan, L., Liu, H., Cao, X., Li, B., Wang, Z., Zhao, X., Yu, H., Wang, F., Ma, W., Huang, J., & Fan, G. (2021). Genomic insights into the fast growth of paulownias and the formation of *Paulownia* witches' broom. *Molecular Plant*, 14(10), 1668-1682. http://dx.doi.org/10.1016/j.molp.2021.06.021. PMid:34214658.

Chagné, D., Lin-Wang, K., Espley, R., Volz, R., How, N., Rouse, S., Brendolise, C., Carlisle, C., Kumar, S., Silva, N., Micheletti, D., McGhie, T., Crowhurst, R., Storey, R., Velasco, R., Hellens, R., Gardiner, S., & Allan, A. (2013). An ancient duplication of apple MYB transcription factors is responsible for novel red fruit-flesh phenotypes. *Plant Physiology*, 161(1), 225-239. http://dx.doi.org/10.1104/pp.112.206771. PMid:23096157.

Chen, W., Gong, L., Guo, Z., Wang, W., Zhang, H., Liu, X., Yu, S., Xiong, L., & Luo, J. (2013). A novel integrated method for large-scale detection, identification, and quantification of widely targeted metabolites: application in the study of rice metabolomics. *Molecular Plant*, 6(6), 1769-1780. http://dx.doi.org/10.1093/mp/sst080. PMid:23702596.

Davies, K. M., Albert, N. W., & Schwinn, K. E. (2012). From landing lights to mimicry: the molecular regulation of flower colouration and mechanisms for pigmentation patterning. *Functional Plant Biology*, 39(8), 619-638. http://dx.doi.org/10.1071/FP12195. PMid:32480814.

Du, H., Lai, L., Wang, F., Sun, W., Zhang, L., Li, X., Wang, L., Jiang, L., & Zheng, Y. (2018). Characterisation of flower colouration in 30 *Rhododendron* species via anthocyanin and flavonol identification and quantitative traits. *Plant Biology*, 20(1), 121-129. http://dx.doi.org/10.1111/plb.12649. PMid:29054107.

Espley, R. V., Hellens, R. P., Putterill, J., Stevenson, D. E., Kutty-Amma, S., & Allan, A. C. (2007). Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. *The Plant Journal*, 49(3), 414-427. http://dx.doi.org/10.1111/j.1365-313X.2006.02964.x. PMid:17181777.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., & Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, 29(7), 644-652. http://dx.doi.org/10.1038/nbt.1883. PMid:21572440.

Hichri, I., Barrieu, F., Bogs, J., Kappel, C., Delrot, S., & Lauvergeat, V. (2011). Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. *Journal of Experimental Botany*, 62(8), 2465-2483. http://dx.doi.org/10.1093/jxb/erq442. PMid:21278228.

Kim, S. J., Seo, G. U., Seo, B. Y., Park, E. J., & Lee, S. C. (2011). Antioxidant activity and DNA damage protective effect of a *Robinia pseudoacacia* L. flower extract. *Korean Journal of Food and Cookery Science*, 27(4), 99-106. http://dx.doi.org/10.9724/kfcs.2011.27.4.099.

Kim, S., Yoo, K. S., & Pike, L. M. (2005). Development of a PCR-based marker utilizing a deletion mutation in the dihydroflavonol 4-reductase (DFR) gene responsible for the lack of anthocyanin production in yellow onions (*Allium cepa*). *Theoretical and Applied Genetics*, 110(3), 588-595. http://dx.doi.org/10.1007/s00122-004-1882-7. PMid:15647922.

Kopustinskiene, D. M., Jakstas, V., Savickas, A., & Bernatoniene, J. (2020). Flavonoids as anticancer agents. *Nutrients*, 12(2), 457. http://dx.doi.org/10.3390/nu12020457. PMid:32059369.

Li, B., & Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, 12, 323. http://dx.doi.org/10.1186/1471-2105-12-323. PMid:21816040.

Lin-Wang, K., Bolitho, K., Grafton, K., Kortstee, A., Karunairetnam, S., McGhie, T. K., Espley, R. V., Hellens, R. P., & Allan, A. C. (2010). An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. *BMC Plant Biology*, 10, 50. http://dx.doi.org/10.1186/1471-2229-10-50. PMid:20302676.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods*, 25(4), 402-408. http://dx.doi.org/10.1006/meth.2001.1262. PMid:11846609.

Ma, X.-L., Chen, J.-Z., Lu, X., Zhe, Y.-T., & Jiang, Z.-B. (2021). HPLC coupled with quadrupole time of flight tandem mass spectrometry for analysis of glycosylated components from the fresh flowers of two congeneric species: *Robinia hispida* L. and *Robinia pseudoacacia* L. *Journal of Separation Science*, 44(7), 1537-1551. http://dx.doi.org/10.1002/jssc.202001068. PMid:33386775.

Maleki, S. J., Crespo, J. F., & Cabanillas, B. (2019). Anti-inflammatory effects of flavonoids. *Food Chemistry*, 299, 125124. http://dx.doi.org/10.1016/j.foodchem.2019.125124. PMid:31288163.

Morita, Y., Ishiguro, K., Tanaka, Y., Iida, S., & Hoshino, A. (2015). Spontaneous mutations of the UDP-glucose: flavonoid 3-O-glucosyltransferase gene confers pale-and dull-colored flowers in the Japanese and common morning glories. *Planta*, 242(3), 575-587. http://dx.doi.org/10.1007/s00425-015-2321-5. PMid:26007684.

Naing, A. H., & Kim, C. K. (2018). Roles of R2R3-MYB transcription factors in transcriptional regulation of anthocyanin biosynthesis in horticultural plants. *Plant Molecular Biology*, 98(1-2), 1-18. http://dx.doi.org/10.1007/s11103-018-0771-4. PMid:30167900.

Nakatsuka, T., Nishihara, M., Mishiba, K., & Yamamura, S. (2005). Two different mutations are involved in the formation of white-flowered gentian plants. *Plant Science*, 169(5), 949-958. http://dx.doi.org/10.1016/j.plantsci.2005.06.013.

Park, N. I., Xu, H., Li, X., Jang, I. H., Park, S., Ahn, G. H., Lim, Y. P., Kim, S. J., & Park, S. U. (2011). Anthocyanin accumulation and expression of anthocyanin biosynthetic genes in radish (*Raphanus sativus*). *Journal of Agricultural and Food Chemistry*, 59(11), 6034-6039. http://dx.doi.org/10.1021/jf200824c. PMid:21548630.

Pei, R., Liu, X., & Bolling, B. (2020). Flavonoids and gut health. *Current Opinion in Biotechnology*, 61, 153-159. http://dx.doi.org/10.1016/j.copbio.2019.12.018. PMid:31954357.

Rédei, K., Osvath-Bujtas, Z., & Veperdi, I. (2008). Black locust (*Robinia pseudoacacia* L.) improvement in Hungary: a review. *Acta Silvatica & Lignaria Hungarica*, 4, 127-132.

Shen, X., Zhao, K., Liu, L., Zhang, K., Yuan, H., Liao, X., Wang, Q., Guo, X., Li, F., & Li, T. (2014). A role for PacMYBA in ABA-regulated anthocyanin biosynthesis in red-colored sweet cherry cv. Hong Deng (*Prunus avium* L.). *Plant & Cell Physiology*, 55(5), 862-880. http://dx.doi.org/10.1093/pcp/pcu013. PMid:24443499.

Takos, A. M., Jaffé, F. W., Jacob, S. R., Bogs, J., Robinson, S. P., & Walker, A. R. (2006). Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. *Plant Physiology*, 142(3), 1216-1232. http://dx.doi.org/10.1104/pp.106.088104. PMid:17012405.

Wang, J., Zhang, L., Liao, Z., Zhang, Y., Qiu, Q., & Sun, Li. (2014a). The selection of reference genes for real-time quantitative PCR normalization in black locust (*Robinia pseudoacacia*). *Linye Kexue*, 50(9), 167-172.

Wang, L., Dai, S., Jin, X., Huang, H., & Hong, Y. (2014b). [Advances in plant anthocyanin transport mechanism]. *Chinese Journal of Biotechnology*, 30(6), 848-863. PMid:25212003.

Wang, L., Zhang, M., & Hu, Q.-H. (2006). Nutrient and functional components in the flower of Robinia pseudoacacia and their exploitation. *Shipin Kexue*, 27(2), 274-276.

Wang, Y. (2019). *Study on honey-derived traits of six Robinia pseudoacacia germplasms*. Tai'an City, Shandong Province, China: Shandong Agricultural University.

Yao, W., Li, C., Lin, S., Wang, J., Zhou, B., & Jiang, T. (2020). Transcriptome analysis of salt-responsive and wood-associated NACs in *Populus simonii* × *Populus nigra*. *BMC Plant Biology*, 20(1), 317. http://dx.doi.org/10.1186/s12870-020-02507-z. PMid:32631231.

Zhang, B., Chopra, D., Schrader, A., & Hülskamp, M. (2019). Evolutionary comparison of competitive protein-complex formation of MYB, bHLH, and WDR proteins in plants. *Journal of Experimental Botany*, 70(12), 3197-3209. http://dx.doi.org/10.1093/jxb/erz155. PMid:31071215.

Zhang, Y., Zhang, Y., Duan, X., Liu, X., Yuan, S., Han, J., & Cheng, Y. (2020). Anthocyanins in tree peony (*Paeonia suffruticosa*) and their relationship with flower color. *Horticultural Science and Technology*, 38(6), 776-784.

Zhao, L., Gao, L., Wang, H., Chen, X., Wang, Y., Yang, H., Wei, C., Wan, X., & Xia, T. (2013). The R2R3-MYB, bHLH, WD40, and related transcription factors in flavonoid biosynthesis. *Functional & Integrative Genomics*, 13(1), 75-98. http://dx.doi.org/10.1007/s10142-012-0301-4. PMid:23184474.

Supplementary Material

Supplementary material accompanies this paper.

Figure S1.

Table S1.

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