Comparative transcriptome analysis reveals key genes associated with pigmentation in radish (*Raphanus sativus* L.) skin and flesh

Jifang Zhang¹,²,4*, Jian Zhao³,4, Qunyun Tan¹,², Xiaojun Qiu¹,² & Shiyong Mei¹,²

Radish (*Raphanus sativus*) is an important vegetable worldwide that exhibits different flesh and skin colors. The anthocyanins responsible for the red and purple coloring in radishes possess nutritional value and pharmaceutical potential. To explore the structural and regulatory networks related to anthocyanin biosynthesis and identify key genes, we performed comparative transcriptome analyses of the skin and flesh of six colored radish accessions. The transcript profiles showed that each accession had a species-specific transcript profile. For radish pigmentation accumulation, the expression levels of anthocyanin biosynthetic genes (*RsTT4*, *RsC4H*, *RsTT7*, *RsCCOAMT*, *RsDFR*, and *RsLDOX*) were significantly upregulated in the red- and purple-colored accessions, but were downregulated or absent in the white and black accessions. The correlation test, combined with metabolome (PCC > 0.95), revealed five structural genes (*RsTT4*, *RsDFR*, *RsCCOAMT*, *RsF3H*, and *RsBG8L*) and three transcription factors (*RsTT8-1*, *RsTT8-2*, and *RsPAR1*) to be significantly correlated with flavonoids in the skin of the taproot. Four structural genes (*RsBG8L*, *RsDFR*, *RsCCOAMT*, and *RsLDOX*) and nine transcription factors (*RsTT8-1*, *RsTT8-2*, *RsbHLH57*, *RsbPAR2L*, *RsbHLH113L*, *RsbGR3L*, *RsbMYB24*, and *RsbMYB34L*) were found to be significantly correlated with metabolites in the flesh of the taproot. This study provides a foundation for future studies on the gene functions and genetic diversity of radish pigmentation and should aid in the cultivation of new valuable radish varieties.

Flavonoids are plant polyphenolic secondary metabolites that are widespread in the plant kingdom. Flavonoids can be classified into many subgroups based on their chemical structures and modifications, including flavanones, flavones, isoflavonoids, flavanols, anthocyanins, and flavonols. Anthocyanins are the major pigment metabolites of flavonoid compounds. They are abundant in the flowers, fruits, seeds, and leaves of many plant species and play important roles in plant protection and reproduction. They can help plants attract pollinators and seed dispersers by stimulating red, purple, and blue pigments in plant tissues. In recent years, there has been increased interest in anthocyanins because of their wide use as natural commercial food pigments and their potential health benefits.

Flavonoid biosynthesis and regulatory pathways have been extensively described. Enzymes, such as CHS, F3H, F3′H, F3′5′H, and LDOX/ANS, are involved in flavonoid biosynthesis and may act as metabolic hubs that influence the overall efficiency, specificity, and regulation of these pathways. DNA-binding R2R3 MYB transcription factors (TFs), MYC-like basic helix-loop-helix (bHLH), and WD40-repeat proteins interact in the regulation of flavonoid biosynthesis. Plant hormones and environmental factors also play important roles in the regulation of anthocyanin biosynthesis. Nevertheless, the mechanisms that regulate anthocyanin metabolism in different plant species and tissues require further investigation.

Radishes (*Raphanus sativus*) are common vegetables worldwide that possess a wide variety of colors, shapes, and sizes. The pigments in radish taproots have health-promoting properties. Based on anthocyanin production,

1 Institute of Bast Fiber Crops, Chinese Academy of Agricultural Science, Changsha, China. 2 Center for Southern Economic Crops, Chinese Academy of Agricultural Science, Changsha, China. 3 Novogene Bioinformatics Institute, Beijing, China. 4 These authors contributed equally: Jifang Zhang and Jian Zhao. *email: smilehome@163.com; hbvegbt@163.com
radishes can be categorized into those that have anthocyanins in their skin and flesh, and those that have anthocyanins only in their skin. Several varieties with different skin and/or flesh taproot colors (e.g., white, green, red, purple, and black) are widely cultivated and consumed. However, the mechanisms that regulate anthocyanin catabolism in radish have not received much research attention.

Fortunately, correlation and clustering analyses based on transcript and metabolite information have been used to further build networks between genes and metabolites in many plants, including Arabidopsis30–32, potato (Solanum lycopersicum)33–34, grape (Vitis vinifera)27, and Actinidia arguta35. The integration of "omics" datasets (i.e., transcriptomes and metabolomes) is considered a useful approach for discovering and identifying potential genes that regulate the determination of radish pigmentation.

In this study, RNA sequencing (RNA-seq) analysis using the Illumina platform was used to study the genetic control of the differential accumulation of anthocyanins. By combining our previous metabolite datasets36 in a bioinformatics analysis, the major metabolic pathways related to radish taproot pigmentation were deduced, and candidate genes targeting the regulation of radish pigmentation were examined.

**Results**

**Anthocyanin contents of radish taproot skin and flesh.** Six radish accessions (Fig. 1) with various skin and flesh colors were used for the anthocyanin content assay. The skin of the radish taproot (Fig. 2), ZJL, which is purple in color, exhibited the highest anthocyanin content, followed by the dark red TXH and the light red MSH. The skin of SZB, black radish, and XLM exhibited trace or undetectable contents. In terms of flesh color (Fig. 2), TXH, which has dark red flesh, showed the highest anthocyanin content, followed by the red-fleshed XLM and the purple-fleshed ZJL. The white flesh of SZB had no detectable anthocyanin content. The purple skin of ZJL had a higher anthocyanin content than the dark red flesh of TXH.

**RNA sequencing and assembly.** The six radish accessions (Fig. 1) with various skin and flesh colors were passed through RNA-seq and analysis, generating a total of 1,695,680,738 raw reads (Table 1). After adaptor sequence trimming and filtering out of low-quality reads, 167,174,278 (98.55%) clean reads were obtained with an average GC content of 46.74% and a Q20 average base quality score of 97.79%. A total of 250.71 Gb of high-quality reads were obtained for the radish samples. Of these clean reads, 68.62–87.7% were mapped to the reference genome WK10039, and 62.3–80.2% were uniquely mapped to WK10039. Furthermore, 31.15–40.09% of the clean reads were mapped to the positive strands, and 31.14–40.15% were mapped to the negative strands (Table S2).

**Differential gene expression levels.** Principal component analysis (PCA) was used to assess inter-group differences and sample duplication within groups. In the PCA plot, 10 samples from the six accessions clustered into six independent groups, suggesting that each group had a relatively distinct transcript profile (Fig. 3A). Group 1 included white-skinned and white-fleshed taproots of the SZB accession (WST and WFT), and group 2 included green-skinned and red-fleshed taproots of the XLM accession (XLMST and XLMFT). The dark red-skinned and dark-red-fleshed taproots of the TXH accession (RST and RFT) clustered in group 3, and the purple-skinned and purple-fleshed taproots of the ZJL accession (ZJLST and ZJLFT) clustered in group 6. Accessions with the same colored skin and flesh clustered together.

To understand the molecular basis and predict the candidate genes responsible for color polymorphisms in radish, differentially expressed genes (DEGs) were identified by pairwise comparisons of the expression levels between colored and white samples. The transcriptome analysis revealed that 19,576 (10,721 upregulated and 8855 downregulated), 22,319 (12,143 upregulated and 10,176 downregulated), 15,776 (8576 upregulated and 7199 downregulated), and 25,105 (14,562 upregulated and 10,543 downregulated) DEGs had at least a two-fold change in the skin of TXH, black radish, XLM, MSH, and ZJL, respectively, compared to SZB (Fig. 3B). There were 15,787 (8452 upregulated and 7335 downregulated), 17,474 (9358 upregulated and 8116 downregulated), and 25,105 (14,562 upregulated and 10,543 downregulated) DEGs had at least a two-fold change in the skin of TXH, black radish, XLM, MSH, and ZJL, respectively, compared to SZB (Fig. 3B). There were 15,787 (8452 upregulated and 7335 downregulated), 17,474 (9358 upregulated and 8116 downregulated), and 25,105 (14,562 upregulated and 10,543 downregulated) DEGs had at least a two-fold change in the skin of TXH, black radish, XLM, MSH, and ZJL, respectively, compared to SZB (Fig. 3B). There were 15,787 (8452 upregulated and 7335 downregulated), 17,474 (9358 upregulated and 8116 downregulated), and 25,105 (14,562 upregulated and 10,543 downregulated) DEGs had at least a two-fold change in the skin of TXH, black radish, XLM, MSH, and ZJL, respectively, compared to SZB (Fig. 3B). There were 15,787 (8452 upregulated and 7335 downregulated), 17,474 (9358 upregulated and 8116 downregulated), and 25,105 (14,562 upregulated and 10,543 downregulated) DEGs had at least a two-fold change in the skin of TXH, black radish, XLM, MSH, and ZJL, respectively, compared to SZB (Fig. 3B). There were 15,787 (8452 upregulated and 7335 downregulated), 17,474 (9358 upregulated and 8116 downregulated), and 25,105 (14,562 upregulated and 10,543 downregulated) DEGs had at least a two-fold change in the skin of TXH, black radish, XLM, MSH, and ZJL, respectively, compared to SZB (Fig. 3B).
transcripts with at least a two-fold change in the flesh of TXH, XLM, and ZJL, respectively, compared to SZB (Fig. 3B). Additionally, the Venn diagram analysis showed that 6024 and 5333 DEGs were considerably differentially expressed in the skin and flesh in the pairwise comparisons, respectively (Fig. 3C,D).

A Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was conducted to identify pathway-related DEGs in the colored skin and flesh of radish. A total of 111 pathways were enriched (Supplementary Table S3). The “biosynthesis of amino acids”, “glyoxylate and dicarboxylate metabolism”, “flavonoid biosynthesis”, “photosynthesis-antenna proteins”, “glycine, serine, and threonine metabolism”, and “carbon metabolism” pathways were significantly enriched in RS vs. WS (Fig. 3A). The “plant-pathogen interaction” pathway was significantly enriched in the skin of MSH (Fig. 3B). The “cyanoamino acid metabolism”, “glyoxylate and dicarboxylate metabolism”, “carbon fixation in photosynthetic organisms”, and “carbon metabolism” pathways were significantly enriched in the skin of XLM (Fig. 3D). DEGs related to the “ribosome” and “ribosome biogenesis in eukaryotes” pathways were significantly enriched in the flesh of XLM (Fig. 3G).

Table 1. Details of the raw data and clean data of all radish samples of various colors. WST: transcript data of SZB with white taproot skin, WFT: transcript data of SZB with white flesh, RST: transcript data of TXH with dark red taproot skin, RFT: transcript data of TXH with dark red flesh, BST: transcript data of black radish, XLMST: transcript data of Xinlimei with green taproot skin, XLMFT: transcript data of Xinlimei with red taproot flesh, PST: transcript data of MSH with light-red taproot skin, ZJLST: transcript data of ZJL taproot skin, ZJLFT: transcript data of ZJL taproot flesh.

| Sample     | Raw reads | Clean reads | Clean bases (G) | Q20avg (%) | GC_pct (%) |
|------------|-----------|-------------|-----------------|------------|------------|
| WST        | 176,281,664 | 173,933,900 | 26.1            | 97.79      | 46.74      |
| WFT        | 177,066,062 | 174,510,406 | 26.18           | 42.12      | 46.74      |
| RST        | 168,928,392 | 166,285,974 | 24.95           | 97.79      | 46.74      |
| RFT        | 168,355,314 | 165,648,598 | 24.85           | 97.79      | 46.74      |
| BST        | 170,937,186 | 168,309,646 | 25.25           | 97.79      | 46.74      |
| XLMST      | 163,260,394 | 160,683,094 | 24.1            | 97.79      | 46.74      |
| XLMFT      | 163,327,024 | 160,571,396 | 24.09           | 97.79      | 46.74      |
| PST        | 154,019,884 | 151,985,018 | 22.8            | 97.79      | 46.74      |
| ZJLST      | 167,115,082 | 165,168,530 | 24.77           | 97.79      | 46.74      |
| ZJLFT      | 186,389,736 | 184,077,716 | 27.62           | 97.79      | 46.74      |
| Total      | 1,695,680,738 | 1,671,174,278 | 250.71       | 97.79      | 46.74      |
Correlation analysis between transcripts and flavonoid derivatives revealed a differential network of flavonoid biosynthesis. To understand the network of flavonoids uncovered by the differential distribution of flavonoid derivatives between colored and white radishes, we conducted correlation tests between the quantitative changes in the metabolites and transcripts. A total of 11 structural genes were enriched in the red and purple radishes compared to the white and black radishes (Fig. 4A,B). Seven genes (RsTT4, 108843267; RsC4H, 108849675; RsHCT, 108812355; RsTT7, 108814778; RsCCOAMT, 108820088; RsDFR, 108826061; and RsLDOX, 108843686) were upregulated in the red and purple radishes, while two genes (RsF3H and RsBAN) did not change significantly. RsFLS (108839020) was downregulated in all of the samples. RsTT5 (108821914) was upregulated in the skin of the five colored radishes, but was downregulated in the flesh of XLM, TXH, and ZJL.

The correlation analysis between transcripts and flavonoid derivatives showed that different interaction modes were responsible for the color diversity of the taproot skin and flesh. For the taproot skin, three genes involved in flavonoid biosynthesis, seven genes involved in phenylpropanoid biosynthesis, and 35 TFs were found to be strongly correlated (Pearson’s correlation coefficient (PCC) > 0.95) with 48 metabolites (Fig. 5A, Table S4). A total of 11 flavonoids were found to be significantly correlated with RsBG8L (108847438, encodes beta-glucosidase 8-like), 10 flavonoids were found to be significantly correlated with RsDFR (108826061, encodes dihydroflavonol-4-reductase), 10 metabolites were found to be significantly correlated with RsCCOAMT (108814129, encodes caffeoyl-CoA O-methyltransferase), six metabolites were found to be significantly correlated with RsTT4...
(108843267, encodes chalcone synthase), and nine flavonoids were found to be significantly correlated with RsF3H (108852668, encodes flavanone 3-hydroxylase). Eleven flavonoids were found to be significantly correlated with RsTT8-1 and RsTT8-2 and nine metabolites were found to be significantly correlated with RsPAR1 (108837711), indicating that these five structural genes and three TFs play critical roles in flavonoid biosynthesis in radish skins.

For taproot flesh, four structural genes were found to be involved in flavonoid biosynthesis, 20 genes were found to be involved in phenylpropanoid biosynthesis, and 57 TFs were strongly correlated (PCC > 0.95) with 93 metabolites (Fig. 5B, Table S5). A total of 29 flavonoids were found to be significantly closely correlated with RsBG8L, 25 flavonoids were found to be significantly closely correlated with RsDFR, 20 metabolites were found to be significantly correlated with RsLDOX (108843686, encodes anthocyanidin synthase), 16 metabolites were found to be significantly correlated with RsCCOAMT, 29 metabolites were found to be significantly correlated with RsbHLH57 (10882212), 28 metabolites were found to be significantly correlated with RsOGR3L (1086444), and 26 metabolites were found to be significantly correlated with RsbHLH113L (108810191), RsMYB24 (108828405), RsMYB34L (108852392), and RsPAR2L (108860104). Twenty-four flavonoids were found to be significantly correlated with RsTT8-2, and 23 flavonoids were found to be significantly correlated with RsTT8-1 and RsbHLH19 (108851776), suggesting that these four structural genes and nine TFs play important roles in flavonoid biosynthesis in radish flesh.

It has been reported that the overexpression of MdMYB24 results in higher anthocyanin content in transgenic apples than in the wild-type controls38. bHLH57 interacts with rdo5 to control ABA biosynthesis and seed dormancy in Arabidopsis36, and AtbHLH19 plays roles in salt tolerance in Arabidopsis36. TT8 has been well characterized in Arabidopsis36,37, Brassica juncea37, and radishes44. AtTT8, a positive regulator, controls its own expression in a feedback regulation involving TTG1 and homologous MYB and bHLH factors, allowing for the strong and cell-specific accumulation of flavonoids in A. thaliana36,37. Natural mutations in two homoeologous TT8 genes control the yellow seed coat trait in B. juncea37. RsTT8 interacts with MYB1 to activate the promoter of RsCHS and RsDFR and so controls pigmentation accumulation in radishes37.
Figure 5. Connection network between the structural genes, TFs, and flavonoid-related metabolites. (A) Network between the genes and flavonoid metabolites in the taproot skin. (B) Network between the genes and flavonoid metabolites in the taproot flesh. Only genes with correlation coefficients > 0.95 are shown. Yellow circles with green frames represent the ID of structural genes involved in flavonoid biosynthesis; the other yellow circles represent the ID of structural genes involved in phenylpropanoid biosynthesis; the red circles indicate the TFs, and the blue boxes represent flavonoid-related metabolites. A and B were visualized using Cytoscape v2.8.2 (https://cytoscape.org/).
Confirmation of the transcriptome data using real-time quantitative PCR (RT-qPCR). Eight genes involved in flavonoid metabolism were selected for RT-qPCR assays to validate the transcriptome datasets obtained from RNA-seq. The results of these assays were consistent with the transcriptome analysis (Fig. 6).

Discussion

Taproot skin and flesh color determine the appearance, quality, and nutritional value of radishes. To understand the molecular basis of the differential distribution of flavonoid derivatives between colored and white radishes, core genes in the flavonoid pathway were studied in detail. The results showed that most of the UniGenes demonstrated significant changes in expression level, regardless of whether they were in the early stage (RsTT4, chalcone synthase; RsC4H, cinnamate-4-hydroxylase; RsTT7, flavonoid 3’-monooxygenase; RsCCOAMT, caffeoyl-CoA O-methyltransferase) or late stage (RsDFR, dihydroflavonol-4-reductase; RsLDOX, anthocyanidin synthase), and showed higher transcript abundances in red and purple radishes than in the white radishes (Figs. 4, 5).

Additionally, core genes involved in upstream phenylpropanoid biosynthesis also affect the productivity of the following flavonoid pathway. These genes, absent or undetectable, are thought to be flux-limiting genes that lead to colorant elimination in white radishes.

It is already known that TT4 catalyzes the first reaction in anthocyanin biosynthesis and helps form the intermediate chalcone, which is the primary precursor of all flavonoid classes. Thus, if TT4 reactions are strongly constrained, then anthocyanin production and that of nearly all other flavonoids could be effectively eliminated. DFR reduces dihydroflavonols to colorless leucoanthocyanidins (apigenin-related glycosides, leucocyanidin, and leucopelargonidin) that are subsequently catalyzed by LDOX to form cyanidin- and pelargonidin-related glycosides, which are then catalyzed by ANS into colored anthocyanidins. Kaempferol-related glycosides are downstream of dihydrokaempferol, catalyzed by FLS, and fluxed into flavone and flavonol biosynthesis. DFR genes have been studied thoroughly in many plant species, including *Medicago truncatula*, *Camellia sinensis*.
Materials and methods
Plant materials. Six advanced radish inbred lines were used in this study: Touxinhong (TXH), with dark red taproot skin and flesh; Xinlimei, with red taproot flesh and green skin; Shizhuangbai (SZB), with white taproot skin and flesh; black radish with black taproot skin and white flesh; Zijinling (ZJL), with purple taproot skin and flesh; and Manshenhong (MSH), with light red taproot skin and white flesh. All plants were grown in pots and increase red pigmentation. TT8 acts a positive regulator of anthocyanin biosynthesis in Arabidopsis siliques and Raphanus sativus. The expression of the AtMYB7 gene can repress the expression of DFR and UGT.

In our study, the expression levels of RsDFR, RsTT4, RsCCOAMT, and RsLODX were undetectable in white radishes, but were abundant in the red-color radishes (Fig. 6). The correlation analysis verified that most flavonoids were significantly correlated with the transcript expression of RsBG8L, RsCCOAMT, and RsDFR both in the skin and in the flesh of the radish taproot. These three genes may lose their biocatalyst function and become pseudogenes due to variations in gene structure or regulation by correlated TFs, which were significantly associated with kaempferol-related glycosides. The radish basic helix-loop-helix TFs, RsTT8-1 and RsTT8-2, were both significantly associated with flavonoids in the skin and flesh of taproot, indicating that these two TFs play crucial roles in the accumulation of pigments in the taproots. It has been reported that RsTT8 interacts with RsMYB1 to activate the promoters of RsCHS and RsDFR, which confirmed the results of our correlation analysis. We here propose that the limitations of flux in upstream reactions and the multishunt process in downstream reactions could lead to the elimination of colored pigmentation in white radishes.
Gene validation and expression analysis. To verify the reliability of the RNA-seq results, eight anthocyanin-related genes were subjected to RT-qPCR with specific primers identified using Primer Premier software (Premier, Canada) (Supplementary Table S1). cDNA synthesis and RT-qPCR were performed as previously described. SYBR Green was used for the detection of PCR products. Actin was used as an internal control for the normalization of gene expression. Three independent biological replicates with three technical replicates each were analyzed by RT-qPCR to ensure reproducibility and reliability. Relative expression levels were estimated using the 2^ΔΔCT method.

Integrative analysis of the metabolome and transcriptome. Pearson's correlation coefficients were calculated for previous metabolome data and transcriptome data integration by Metware Biotechnology (Wuhan, China). The mean of all biological replicates of each cultivar in the metabolome data and the mean value of expression of each transcript in the transcriptome data were calculated. The fold-change of each pigmented radish accession (TXH, XLM, and ZJL) was calculated for both the metabolome and transcriptome data, and then compared with the control accession (SZB with white skin and white flesh). Finally, the coefficients were calculated from the log2fold change of each metabolite and transcript using Excel (Microsoft Corporation, Redmond, WA, USA). Correlations corresponding to a coefficient with PCC > 0.95 were selected (Supplementary Table S4). Metabolome and transcriptome relationships were visualized using Cytoscape v2.8.2.

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Author contributions
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Correspondence and requests for materials should be addressed to J.Z. or S.M.

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