Comparative transcriptome analyses reveal genes related
to pigmentation in the petals of a flower color variation cultivar
of Rhododendron obtusum

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Abstract
Background Rhododendron is an important woody ornamental plant, and breeding varieties with different colors is a key research goal. Although there have been a few reports on the molecular mechanisms of flower colors and color patterning in Rhododendron, it is still largely unknown what factors regulate flower pigmentation in Rhododendron.

Methods and results In this study, the flower color variation cultivar ‘Yanzhi Mi’ and the wild-type (WT) cultivar ‘Dayuan-yangjin’ were used as research objects, and the pigments and transcriptomes of their petals during five flower development stages were analyzed and compared. The results showed that derivatives of cyanidin, peonidin and pelargonidin might be responsible for the pink color of mutant petals and that the S2 stage was the key stage of flower color formation. In total, 412,910 transcripts and 2780 differentially expressed genes (DEGs) were identified in pairwise comparisons of WT and mutant petals. GO and KEGG enrichment analyses of the DEGs showed that ‘DNA-binding transcription factor activity’, ‘Flavonoid biosynthesis’ and ‘Phenylpropanoid biosynthesis’ were more active in mutant petals. Early anthocyanin pathway candidate DEGs (CHS3-CHS6, CHI, F3Hs and F3’H) were significantly correlated and were more highly expressed in mutant petals than in WT petals in the S2 stage. An R2R3-MYB unigene (TRINITY_DN55156_c1_g2) was upregulated approximately 10.5-fold in ‘Yanzhi Mi’ petals relative to ‘Dayuan-yangjin’ petals in the S2 stage, and an R2R3-MYB unigene (TRINITY_DN59015_c3_g2) that was significantly downregulated in ‘Yanzhi Mi’ petals in the S2 stage was found to be closely related to Tca MYB112 in cacao.

Conclusions Taken together, the results of the present study could shed light on the molecular basis of anthocyanin biosynthesis in two Rhododendron obtusum cultivars and may provide a genetic resource for breeding varieties with different flower colors.

Keywords Rhododendron · Anthocyanin biosynthesis · Transcriptome · Flower coloration · R2R3-MYB

Introduction
Rhododendron is not only the largest genus of the family Ericaceae but is also an important ornamental woody plant group that includes approximately 1000 species and thousands of commercial hybrids [1]. Rhododendron plants are familiar ornamental shrubs that are widespread around the world and have beautiful flowers. Flower color is an important ornamental trait that greatly affects the economic value of Rhododendron. There is a remarkably broad range of Rhododendron flower colors, including white, red, pink, purple, yellow, green, and blue [2]. Previous studies have shown that many factors (cell shape, copigmentation, pH, environmental conditions, and so forth) affect petal coloration, among which the pigment composition is the most important [3]. The petals of Rhododendron flowers contain flavonols and anthocyanins as their major pigments [4]. The main flavonols of Rhododendron petals are quercetin, kaempferol, and myricetin, although many others exist [5–7]. The flavonol composition and content have some effect on flower color by copigmentation, but might not play a major role in coloration [8]. The major anthocyanidins

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of *Rhododendron* petals are cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin [4, 9, 10]. The anthocyanin composition and content are fundamental elements that determine *Rhododendron* flower colors [4, 8, 11]. Therefore, the study of anthocyanin biosynthesis in the petals of *Rhododendron* plants and the related regulatory mechanisms are important for elucidating the formation of different flower colors in *Rhododendron*.

The anthocyanin biosynthesis pathway is conserved in higher plants [12, 13]. The biosynthesis of anthocyanins and the associated regulatory mechanisms mediated by transcription factors have been carefully studied in most model plants [14]. Enzyme encoding structural genes, including the phenylalanine-ammonia lyase (PAL), 4-coumaryl-CoA ligase (4CL), chalcone isomerase (CHI), flavonoid 3′-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase (F3′5′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose:flavonoid 3′-O-glucosyltransferase (UGFT), are involved in anthocyanin biosynthesis [15, 16]. The expression of these structural genes is regulated at the transcriptional level by a complex of transcription factors including R2R3-MYB, basic helix–loop–helix (bHLH), and WD40 proteins (referred to as the MBW complex) that can bind to the promoters of these structural genes [17, 18]. Among these transcription factors, R2R3-MYB transcription factors play a major role in determining the spatiotemporal expression of anthocyanin biosynthetic genes [19, 20].

To date, there have been a few reports on the molecular mechanisms of flower colors and color patterning in *Rhododendron*. CHS and DFR were cloned from *R. simsii* hybrids and the expression of these two genes was examined in eight azalea cultivars, the results showed that there was no significant correlation between the flower color phenotype and expression levels of the CHS and DFR [21, 22]. The main enzymes in the anthocyanin biosynthesis pathway in azalea include CHS, CHI, F3H, F3′H, F3′5′H, DFR, ANS, and flavonol synthase (FLS) [17]. The genes encoding these biosynthetic enzymes were isolated from evergreen azalea *R. × pulchrum* ‘Sweet ‘Oomurasaki’, and expression analysis showed that only F3′5′H gene expression was strongly correlated with the accumulation of total anthocyanins in petals [23]. A comparative study between purple-flowered ‘Oomurasaki’ and its red-flowered mutant showed that ‘Oomurasaki’ contained anthocyanidins from both the cyanidin and delphinidin series, whereas the red flower mutant contained only pigments from the cyanidin series. The transcript levels of F3′5′H in the mutant were 0.14-fold those in ‘Oomurasaki’, suggesting that low transcript levels of F3′5′H in the red-flowered mutant resulted in no accumulation of delphinidin-derived anthocyanin [9]. Despite these reports, it is still largely unknown what factors regulate flower pigmentation in *Rhododendron*.

Our laboratory bred the flower color variant cultivar ‘Yanzhi Mi’ (pink petals), which was derived from a bud variation of *Rhododendron obtusum* ‘Dayuanyangjin’ (white petals with pink stripes). The only difference from ‘Dayuanyangjin’ observed in ‘Yanzhi Mi’ is a difference in flower color, and the genetic background is otherwise the same as that of ‘Dayuanyangjin’; thus, ‘Yanzhi Mi’ provides ideal experimental material for studying the mechanism of *Rhododendron* petal coloration. In the current study, we qualitatively and quantitatively characterized and compared pigmentation in the petals of the mutant ‘Yanzhi Mi’ and the corresponding wild-type (WT), ‘Dayuanyangjin’, in five stages of flower development. Then, we used the Illumina sequencing platform to conduct a transcriptome sequencing analysis of mixed RNA extracted separately from the petals of ‘Yanzhi Mi’ and ‘Dayuanyangjin’ in the same five stages of flower development. After analyzing the data, we identified some key candidate genes related to anthocyanin biosynthesis in *R. obtusum*. These transcriptome sequences may provide a valuable genomic resource for better understanding the molecular mechanisms of color formation in *R. obtusum*.

### Materials and methods

#### Plant materials

*R. obtusum* ‘Dayuanyangjin’ (D) and ‘Yanzhi Mi’ (Y) were grown in a greenhouse at the Jiangsu Academy of Agricultural Sciences (Nanjing, China, 118.881E, 32.039 N). In March 2019, flowers in five developmental stages [closed buds (S1 stage), buds showing color at the top but with scales still present (S2 stage), the initial flowering stage (S3 stage), the full flowering stage (S4 stage) and the last flowering stage (S5 stage)] were sampled from ten plants of each cultivar (Fig. 1). The petals were immediately cut off, frozen in liquid nitrogen, and then stored at −80 °C for pigment measurement and RNA-seq analyses. Three biological replicates were analyzed for each sample.

#### Extraction and measurement of pigment

Anthocyanins and flavonoids were extracted and measured according to a previous study [24]. One gram of fresh azalea petal tissue was immersed in 5 ml of an extraction mixture consisting of acetonitrile, water and formic acid (5:5:1, v:v:v) at 4 °C for 24 h, and extraction was then performed by ultrasonication at ambient temperature for 30 min. The samples were subsequently filtrated, and the residue was extracted with 5 ml of extraction solution for 30 min and filtered again. After two filtration steps, the two filtrate samples were combined and concentrated to 5 ml under a
nitrogen gas stream. The concentrated extract was passed through a SPEC18 Sep-pak (GRAE company, USA), and the eluent was used for testing. All samples were analyzed on an Agilent 1260 ultra-high performance liquid chromatography (UHPLC) system coupled with a 6530 quadrupole-time of flight (Q-TOF) mass spectrometer (HPLC-Q-TOF-MS) operating in positive ion mode. Chromatographic separation was performed in an Agilent Poroshell 120 SB-Aq column (4.6 × 100 mm, 2.7 μm). Separation was carried out via the following 50 min multistep linear gradient using a mobile phase consisting of (A) 1% v aqueous formic acid in water and (B) acetonitrile: from 0 to 20 min, increase from 5% B to 25% B; from 20 to 40 min, increase 25% B to 100% B; and then maintenance of 100% B for 10 min, not including post-time. The flow rate was 0.3 mL min⁻¹, the column temperature was 35 °C, the injection volume was 20 μL, and detection was performed at 530 nm and 254 nm. The source temperature was set at 350 °C, capillary voltage at + 4.0 kV, N2 drying gas flow at 10 mL min⁻¹, nebulizer pressure at 50 psi, and fragmentor voltage at 175 V. The collision energy was set at 25 V for MS/MS analysis.

The compounds in the sample extracts were identified by comparison with the retention times of standards. The characteristics of the UV–Vis spectra of peaks and the mass spectrometric information were analyzed using a MassHunter B0.05.0 Workstation (Agilent, USA). The relative contents of anthocyanins and flavonoids were calculated from the peak areas of the samples based on the intensity of the corresponding standard compounds, including cyanidin, pelargonidin, peonidin, cyanidin-3-rutinoside, peonidin 3,5-diglucoside, cyanidin-3-glucoside, kaempferol, quercetin, quercetin-3-glucoside, Isorhamnetin and proanthocyanidin. For compounds lacking the corresponding standards, quantification was performed using similar compounds. The mean values and standard deviations (SDs) were calculated from three biological replicates.

RNA extraction, cDNA library construction and RNA-seq

Total RNA was isolated from 1 mg of mixed petal power from ten plants using a TRIzol kit according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). The RNA concentration and purity were assessed using a Qubit RNA Assay Kit on a Qubit 2.0 Fluorometer (Life Technologies, CA, USA) and a NanoDrop2000 spectrophotometer (IMPLEN, CA, USA), respectively. RNA integrity was examined using an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) according to an RNA integrity number (RIN) > 8.0. Three biological replicates were included for each flower development stage in each variety. Library construction and RNA-seq analyses were performed by Gene-pioneer Biotechnology Corporation (Nanjing, China) using the Illumina HiSeqTM 2500 platform. A total of 30 RNA-seq libraries were generated (Table S1). All sequencing data were deposited in NCBI Sequence Read Archive under accession number SRP304986.
Transcriptome assembly and gene annotation

After removing the poor-quality reads (adaptor reads, ambiguous nucleotides and low-quality reads) [25], the clean reads were assembled using Trinity software as previously described for de novo transcriptome assembly without a reference genome [26]. The assembled unigenes were searched against public databases, including the NCBI, NR, COG, SwissProt, Pfam, GO, KOG and KEGG database.

DEG analysis

The gene expression levels of unigenes were estimated using the FPKM method in RSEM software [27]. Clean data from each sample were mapped back onto the assembled transcripts using bowtie2 software [28], and the normalized expression values RPKM (reads per kilobase per million mapped reads) of each unigene in the 30 libraries were used to represent gene expression levels [29]. Differential expression analysis was carried out with DESeq2 [30]. Threshold criteria based on the FDR statistical method were used to determine significant differences in gene expression, and the normalized expression values RPKM were compared according to the thresholds of a P-value ≤ 0.01 and a log2(FC)≥ 1 based on an FDR value ≤ 0.05. DEGs (FDR value ≤ 0.05 and log2(FC)≥ 1) were determined and then analyzed through GO and KEGG pathway enrichment analysis using GoSeq and KOBAS (2.0), respectively [31, 32].

Phylogenetic analysis

A phylogenetic tree of selected R2R3-MYB transcription factors was constructed using MEGA 5.1 software via the neighbor-joining method with bootstrap analysis of 1000 replicates [33].

Validation of DEGs by quantitative real-time PCR

qPCR analysis was performed using a SYBR® Premix Ex TaqTM Kit (TaKaRa, Dalian, China) and an ABI 7500 Real-Time PCR system (Applied Biosystems, CA, USA). The RNA samples used for the qRT-PCR assays were the same samples employed for sequencing. Total RNA (1 μg) was used to synthesize cDNA with the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China) following the recommended procedures. All qRT-PCR assays were performed in a total volume of 20 μL containing 10 μL of SYBR Master Mix, 2 μL of cDNA template, 0.4 μL of 10 μM forward and reverse primers and 7.2 μL of dd H2O. The cycling conditions were as follows: one cycle of 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s, 60 °C for 15 s, and 72 °C for 30 s. The housekeeping gene Actin was used as the control gene [34]. All primers used in these assays are listed in Table S2.

The specificity of each primer pair was checked by agarose gel electrophoresis and melting curve analysis. The relative expression levels of the genes were calculated using the \(2^{-\Delta\Delta CT} \) formula [35]. Both biological and technical triplicates of each sample were analyzed.

Statistical analysis

The statistical analyses of anthocyanin and flavonoid contents were conducted with the one-way ANOVA LSD test using the IBM SPSS Statistics (version 19) statistical software. P values < 0.05 were considered significant. Correlation tests were performed by calculating the Pearson correlation coefficient (Pearson’s r) with a two-tailed test. Correlations were considered positive when Pearson’s r > 0.65 and P < 0.05.

Results

Qualitative and quantitative pigments analyses

The total anthocyanin contents of ‘Yanzhi Mi’ and ‘Dayuanyangjin’ petals were similar in the S1 stage. In the other four floral development stages, total anthocyanins accumulated to significantly higher levels in ‘Yanzhi Mi’ petals, especially in the S2 stage; the anthocyanin contents of ‘Yanzhi Mi’ petals were 35.9 times higher than those of ‘Dayuanyangjin’ petals in the S2 stage (Fig. 2A). In ‘Yanzhi Mi’ petals, the anthocyanin contents were highest in the S2 stage, decreased rapidly in the S3 stage, and remained unchanged in the S4 and S5 stages. In contrast, the anthocyanin contents of ‘Dayuanyangjin’ petals were highest in the S1 stage, decreased rapidly in the S2 stage, and remained unchanged until the S5 stage. The flavonoid contents in ‘Dayuanyangjin’ petals were higher than those in ‘Yanzhi Mi’ petals in the S1 stage and were similar in the other four floral development stages (Fig. 2A). The results indicated that anthocyanins might play vital roles in pink flower color formation of ‘Yanzhi Mi’.

Therefore, we further identified the anthocyanin composition of the petals of ‘Yanzhi Mi’ and ‘Dayuanyangjin’. In total, seven anthocyanins were identified in the petals of the two cultivars, which included cyanidin-3-glucoside-5-xyloside, cyanidin-3-rutinoside, cyanidin-3-glucoside, cyanidin-3-xyloside, peonidin -3-glucoside-5-xyloside, peonidin 3,5-diglucoside and pelargonidin-3-dipharmnoside. Among the seven anthocyanins, pelargonidin-3-dipharmnoside, cyanidin-3-glucoside-5-xyloside and cyanidin-3-xyloside are the three most abundant anthocyanins in the petals of ‘Yanzhi Mi’ in the S2 stage (Fig. 2B and Table S3). These results suggested that the derivatives of cyanidin, peonidin and pelargonidin might be responsible for the pink coloration of ‘Yanzhi Mi’ petals.
Based on the analysis of anthocyanins, we further investigated petal transcriptome variation in ‘Yanzhi Mi’ and ‘Dayuanyangjin’ in five floral development stages by using Illumina technology. Following data cleaning and quality checking, 722,321,099 reads with Q30 values ≥ 90.00% were obtained from the 30 libraries. Among the clean reads, > 96% showed quality scores at the Q20 level (Table S1). The raw sequencing data have been deposited in the NCBI database and can be accessed in the Short Read Archive (SRP304986).

The total length of the clean reads was 216.70 Gb, which was equivalent to ~310-fold coverage of the genome of \textit{R. obtusum} (approximately 0.7 Gb). All clean reads were merged and de novo assembled using Trinity platform software, resulting in the generation of 412,910 transcripts with an average length of 1276.10 bp and an N50 length of 1881 bp (Table S4). Most of the reads could be mapped back to the assembled transcripts, and the total length of all transcripts was approximately 527 Mb. These transcripts were further subjected to cluster and assembly analyses, which resulted in 137,018 unigenes with an average length of 1069.22 bp and an N50 value of 1549 bp, among which 34.19% of the unigenes (46,853) were more than 1 kb in length (Table S4).

Sequence similarity searches were carried out against protein sequences available in various databases using the BLASTX algorithm with an E value threshold of 1e\(^{-10}\). Among the 137,018 unigenes, 77,578 (56.6%), 45,902 (33.5%), 42,496 (31.0%), 28,005 (20.4%), 18,018 (13.2%), 49,672 (36.3%) and 44,816 (32.7%) were blasted into the NCBI and database of Non-Redundant Protein Sequences (NR), Gene Ontology (GO), Eukaryotic Ortholog Groups (KOG), Cluster of Orthologous Groups of Proteins (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), Swiss-Prot protein (SwissProt), Protein Family (Pfam),
upregulated DEGs were observed in six GO categories (p-value ≤ 0.05) were identified in Y-S2 vs D-S2, and ‘binding’, ‘cell’, and ‘cellular process’ were the most highly represented GO terms in MF, CC, and BP, respectively, among which the most highly enriched terms, ‘cell’, was enriched with 1169 DEGs (Fig. S3A). Significantly upregulated DEGs were observed in six GO categories (‘DNA-binding transcription factor activity; GO:003700’, ‘sequence-specific DNA binding; GO:0043565’, ‘DNA replication initiation; GO:0006270’, ‘flavonoid biosynthetic process; GO:0009813’, ‘MCM complex; GO:0042555’ and ‘naringenin-chalcone synthase activity;GO:0016210’) in the S2 stage (Fig. S3B). Among these GO categories, four (DNA-binding transcription factor activity, sequence-specific DNA binding, flavonoid biosynthetic process and naringenin-chalcone synthase activity) were found to be related to the color of flowers. The GO categories pectin catabolic process (GO:0045490), apoplastic activity (GO:0048046), chitinase activity (GO:0004568) and pectate lyase activity (GO:0030570) were found to more highly enriched among downregulated DEGs in the S2 stage (Fig. S3B).

Analysis of DEGs in five floral development stages

The criteria of a false discovery rate (FDR) ≤ 0.05 and a log2 (FC) ≥ 1 were used as the standards for the pairwise differential expression analysis of the five sample groups (Y-S1 vs D-S1, Y-S2 vs D-S2, Y-S3 vs D-S3, Y-S4 vs D-S4 and Y-S5 vs D-S5) (Fig. S2). A total of 2780 differentially expressed genes (DEGs) were identified in the ‘Yanzhi Mi’ vs ‘Dayuanyangjin’ comparison in the five floral development stages. No DEGs were found in the S1 stage. Totally, 2546 DEGs were identified in the Y-S2 vs D-S2 comparison, including 1327 upregulated and 1219 downregulated genes. The largest numbers of both upregulated DEGs and downregulated DEGs were observed in this comparison. A sharp decline in the total number of DEGs between ‘Yanzhi Mi’ and ‘Dayuanyangjin’ was observed in the S3 stage, when a total of 213 upregulated and 33 downregulated genes were recorded. The total number of DEGs in Y-S4 vs D-S4 (44 DEGs, 31 upregulated and 13 downregulated) was similar to that in Y-S5 vs D-S5 (46 DEGs, 27 upregulated and 19 downregulated), and there were very few DEGs in both of these comparisons (Fig. S2). Overall, the number of upregulated genes identified in ‘Yanzhi Mi’ petals was greater than the number of downregulated genes during floral development. These results indicate that the S2 stage is the key stage for determining the difference in flower color between ‘Yanzhi Mi’ and ‘Dayuanyangjin’.

We conducted GO enrichment and KEGG pathway analyses for illustrating the main biological functions of the DEGs. A GO enrichment analysis can give a description of gene products in terms of their associated Molecular Function (MF), Cellular Component (CC) and Biological Process (BP) [36].

The DEGs were assigned into a total of 53 GO categories, and 1,607 DGEs identified in Y-S2 vs D-S2 were categorized into all 53 GO categories. Forty-two significant enriched GO categories (p-value ≤ 0.05) were identified in Y-S2 vs D-S2, and ‘binding’, ‘cell’, and ‘cellular process’ were the most highly represented GO terms in MF, CC, and BP, respectively, among which the most highly enriched terms, ‘cell’, was enriched with 1169 DEGs (Fig. S3A). Significantly upregulated DEGs were observed in six GO categories

DEGs involved in the anthocyanin biosynthesis pathway and their expression patterns

Qualitative and quantitative pigment analyses of ‘Yanzhi Mi’ and ‘Dayuanyangjin’ petals showed that anthocyanins might play vital roles in petal color variation in ‘Yanzhi Mi’. Therefore, the focus of this study was mainly on the unigenes involved in the anthocyanin biosynthesis pathway. Based on the gene annotation and DEGs analysis, the targeted genes associated with anthocyanins and the related biosynthesis pathways were screened and compared.

According to the results of pigment determination and the GO and KEGG annotation of DEGs, 62 DEGs related to flower color formation were identified (Table 1).

Among these DEGs, 16 candidate anthocyanin structural genes encoding seven putative enzymes were involved in anthocyanin biosynthesis in ‘Yanzhi Mi’ petals. The
synthetic pathways and expression profiles of these genes are shown in Fig. 3A. These genes included CHS (6), CHI (1), F3H (2), F3’H (1), DFR (2), ANS (1), and GT (3), and they exhibited significant differential expression patterns in the petals of ‘Yanzhi Mi’ vs ‘Dayuanyangjin’. We found that except for the GT genes, all of the genes encoding the six enzymes showed similar expression levels in the petals of ‘Yanzhi Mi’ and ‘Dayuanyangjin’ in the S1 stage, and the three genes encoding GTs showed lower expression levels in ‘Yanzhi Mi’ petals than in ‘Dayuanyangjin’ petals. In the S2 stage, the expression levels of thirteen genes (except for GTs) were higher in ‘Yanzhi Mi’ petals than in ‘Dayuanyangjin’ petals. In the S3 stage, thirteen genes showed higher expression levels in ‘Yanzhi Mi’ petals, and 5,3GT (TRINITY_DN64021_c2_g2) was upregulated 2.21-, 6.13-, 5.20- and 5.83-fold in the S2 stage compared to its expression in the S1, S3, S4 and S5 stages, respectively. However, in ‘Dayuanyangjin’ flowers, the expression levels of CHS3, CHS4, CHS5, CHS6, CHI, F3H1, and F3’H were expressed more highly in the S2 stage than in other floral development stages; among these genes F3’H (TRINITY_DN68439_c2_g2) was upregulated 1.14-, 4.22-, 5.20- and 5.83-fold in the S2 stage compared to its expression in the S1, S3, S4 and S5 stages, respectively. In ‘Yanzhi Mi’, many genes in the anthocyanin synthetic pathways showed maximum expression in the S2 stage, after which their expression decreased in each subsequent developmental stage. For example, CHS3, CHS4, CHS5, CHS6, CHI, F3H1, and F3’H were expressed more highly in the S2 stage than in other floral development stages; among these genes F3’H (TRINITY_DN68439_c2_g2) was upregulated 1.14-, 4.22-, 5.20- and 5.83-fold in the S2 stage compared to its expression in the S1, S3, S4 and S5 stages, respectively. However, in ‘Dayuanyangjin’ flowers, the expression levels of ten genes (CHSs, CHI, F3Hs and F3’H) were highest in the S1 stage. For example, F3’H (TRINITY_DN68439_c2_g2) was upregulated 2.21-, 6.13-, 6.24- and 5.93-fold in the S1 stage, respectively, compared to its expression in the S2, S3, S4 and S5 stages.

The correlations of the expression profiles of the above-mentioned 16 genes were detected by Pillet’s method [37], and positive correlations between these genes (Pearson’s r > 0.65, P < 0.05) were identified (Fig. 3B, C). Compared with the gene expression correlation map of ‘Dayuanyangjin’ petals, we found that eight early anthocyanin pathway genes (except for GTs) were more highly correlated and shared more than two positive correlations with other genes in ‘Yanzhi Mi’ petals. Interestingly, a late anthocyanin pathway gene, 3GT1, showed a different regulatory mechanism and shared negative correlations with six early anthocyanin pathway genes.
This correlation pattern clearly suggests that these anthocyanin pathway genes might be coregulated by the same transcription factors. A ternary complex that comprised three groups of transcription factors (R2R3-MYB, bHLH, and WDR) can coordinately regulate the expression of most of the structural genes in the anthocyanin biosynthetic pathway [38]. Therefore, the expression levels of the regulatory factors that potentially control anthocyanin biosynthesis were analyzed. The results showed that the S2 stage is the key stage in which the flower color difference between ‘Yanzhi Mi’ and ‘Dayuanyangjin’ is determined. Thus, we selected the transcription factors that were related to flower color formation and were differentially expressed in the two cultivars in the S2 stage for further analysis. We 35 DEGs encoding candidate transcription factors from the DEG data were identified, including 26 unigenes encoding R2R3-MYBs and 9 encoding bHLHs. Among the 26 predicted MYBs, 14 were highly
expressed in ‘Yanzhi Mi’ (in cluster 2), and 12 were highly expressed in ‘Dayuanyangjin’ (in cluster 1) (Fig. 4A). Five unigenes were differentially expressed with absolute log2 (FC) values > 2, including two downregulated genes (TRINITY_DN62378_c2_g1 and TRINITY_DN59015_c3_g2) and three upregulated genes (TRINITY_DN55156_c1_g2, TRINITY_DN53621_c6_g1 and TRINITY_DN62502_c1_g3). Notably, TRINITY_DN55156_c1_g2 was upregulated approximately 10.5-fold in ‘Yanzhi Mi’ relative to ‘Dayuanyangjin’, and TRINITY_DN59015_c3_g2 was found to be closely related to Tca MYB112 in cacao (Fig. S5) [39]. Among the 9 predicted bHLHs, 8 were highly expressed in ‘Yanzhi Mi’ (in cluster 2), and 1 was highly expressed in ‘Dayuanyangjin’ (in cluster 1) (Fig. 4B). Four unigenes were differentially expressed with absolute log2 (FC) values > 2.0, and they were all upregulated (TRINITY_DN0930_c0_g3, TRINITY_DN44252_c0_g1, TRINITY_DN42619_c0_g1 and TRINITY_DN53618_c2_g2). TRINITY_DN53618_c2_g2 showed particularly high upregulation, with a maximum multiple of 2.43.

**qPCR validation of the DEGs of the flavonoid pathway**

To confirm the transcripts obtained in the sequencing analysis, the reliability of the comparative transcriptional data was further verified. A total of 10 structural gene transcripts and transcription factor transcripts that might be involved in anthocyanin biosynthesis were randomly selected for qPCR test. The relative expression levels of these transcripts in the five floral development stages of ‘Yanzhi Mi’ and the transcripts per million clean tags (TPM) values of these transcripts obtained from the sequencing data from the 15 ‘Yanzhi Mi’ samples are shown in Fig. S6. These results showed that the expression patterns obtained by qPCR were consistent with the digital expression data and indicated that the transcriptomic data used in this study for anthocyanin synthesis-related gene analysis were reliable and highly reproducible.

**Discussion**

Flower color is one of the most important ornamental traits of rhododendrons, and the study of the mechanism underlying the formation of different flower colors is of great significance. The major anthocyanidins in rhododendron petals are cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin [4, 9, 10]. Seven anthocyanins were previously identified in the petals of 30 Rhododendron species, among which the red-flowered species mainly contained 2 cyanidin monoglucosides [8]. A total of 5 anthocyanin compounds were identified in 10 Rhododendron species, and cyanidin derivatives were the major anthocyanins found in most of the red–purple group and in R. triflorum (red flower) [40]. The flowers of R. schlippenbachii Maxim contained 2 anthocyanins, cyanidin-3,5-diglucoside and cyanidin-3-sambubioside, and among 3 different flower colours found in
R. schlippenbachii, red flowers exhibited higher amounts of total anthocyanins than violet flowers, whereas no anthocyanins were detected in white flowers [11]. Previous studies have not clarified the mechanism responsible for pink flowers, but some researchers consider pink coloration to result from a pigment gradient [41]. De Keyser et al. also confirmed that pink coloration in Rhododendron can be observed to result from a lower intensity of red (carmine) pigmentation by means of image analysis [22]. In this study, we compared the pigmentation of the petals of the ‘Yanzhi Mi’ mutant and the corresponding WT ‘Dayuanyangjin’ during floral development. According to HPLC-Q-TOF-MS analysis, ‘Yanzhi Mi’ and ‘Dayuanyangjin’ showed similar contents of flavonoids, but significantly different contents of anthocyanins with the same compositions. During floral development, the pink petals of ‘Yanzhi Mi’ exhibited a higher content of anthocyanins, mainly composed of seven derivatives of cyanidin, pelargonidin and peonidin. These results are consistent with previous reports indicating that the presence of cyanidin and peonidin leads to the formation of red petals [31]. The white petals of ‘Dayuanyangjin’ mainly contained flavonoids, and the trace levels of anthocyanins in the white petals may be responsible for the presence of pink stripes in them.

The biosynthesis of anthocyanins is crucial to expand the range of flower colors. Previous studies have shown that C4H, CHS, F3H, F3′H, DFR and ANS are the key enzymes involved in the biosynthesis of anthocyanins [42]. Flower color regulation is achieved mainly via the coordinated transcriptional control of structural genes [43, 44]. In this study, we identified 16 DEGs in the petal transcriptomes of ‘Yanzhi Mi’ vs ‘Dayuanyangjin’ that encoded seven putative enzymes involved in anthocyanin biosynthesis. By comparing their gene expression profiles during floral development, we found that except for the GTs, all of the other genes encoding the six enzymes showed similar expression levels in the petals of ‘Yanzhi Mi’ and ‘Dayuanyangjin’ in the S1 stage. However, most of the genes showed significantly higher transcript levels in ‘Yanzhi Mi’ petals than in ‘Dayuanyangjin’ petals in the S2 and S3 stages, especially in the S2 stage. The comparative analysis of the petal transcriptomes of ‘Yanzhi Mi’ vs ‘Dayuanyangjin’ showed that from stage S1 to stage S5, the DEGs were mainly concentrated in stage S2, which accounted for 91.6% of the total DEGs. Based on the identification of no DEGs in the S1 stage and the similar anthocyanin contents of ‘Yanzhi Mi’ vs ‘Dayuanyangjin’ petals in this stage, we can conclude that the S2 stage is the key stage for flower color formation in ‘Yanzhi Mi’.

In R. × pulchrum flower buds in the S1 stage, five genes (CHS, CHI, F3H, DFR, and ANS) were expressed, but no anthocyanins were detected. Among these genes, the expression of CHS, F3H, and ANS was highest in the S1 stage and decreased during flower development, whereas CHI and DFR transcripts were expressed at the highest levels in the S2 stage [23]. However, the anthocyanin content was highest at the candle stage (buds showing color at the top but without any remaining scales) [21, 23]. Anthocyanin synthesis and gene expression in R. × pulchrum petals were not coincident. In our study, only two CHS candidate genes (CHS1 and CHS2) showed the highest transcript accumulation in the S1 stage of ‘Yanzhi Mi’ flowers, and other early pathway candidate genes (CHS3-6, CHI, F3Hs, and F3′H) were strongly expressed in ‘Yanzhi Mi’ petals in the S2 stage, after which their expression declined in the subsequent stages of floral development. The maximum expression of later pathway candidate genes (DFRs, ANS and UFGTs) was found in the S3, S4 and S5 stages (Fig. 3A). The lowest content of anthocyanins was detected in the S1 stage in ‘Yanzhi Mi’ flowers, and the highest content of anthocyanins was found in the S2 stage in ‘Yanzhi Mi’ flowers (Fig. 2B). However, in ‘Dayuanyangjin’, the expression levels of all early pathway candidate genes (CHS1-6, CHI, F3Hs, and F3′H) were highest in the S1 stage and decreased in each subsequent developmental stage. Late pathway candidate genes (DFRs, ANS and UFGTs) were highly expressed in the S2, S4 and S5 stages (Fig. 3A). The anthocyanin content of ‘Dayuanyangjin’ petals in the S1 stage, which was highest among the five floral development stages, was similar to that of ‘Yanzhi Mi’ petals in the S1 stage. Combined with the above results, we suggest that in ‘Yanzhi Mi’ and ‘Dayuanyangjin’, anthocyanin synthesis and gene expression in petals are coincident in the key stage of flower color formation, the S2 stage, which is different from the situation in R. × pulchrum flowers; flower color formation in ‘Yanzhi Mi’ and ‘Dayuanyangjin’ subsequently begins to diverge in the S2 stage. A combination of early pathway genes (CHS, CHI, F3H, and F3′H) are correlated with the differentiation between the pink flowers of ‘Yanzhi Mi’ and the white flowers of ‘Dayuanyangjin’. Therefore, we speculated that CHS, CHI, F3H and F3′H were the most likely candidates responsible for flower coloration in ‘Yanzhi Mi’.

R2R3-MYB, bHLH and WD40 proteins are the main transcription factors responsible for regulating the expression of structural genes in the anthocyanin biosynthesis pathway [26]. Often, these transcription factors form a complex (the MYB-bHLH-WDR, or MBW, complex) that can coordinately activate or repress the expression of a set of target genes in the anthocyanin biosynthesis pathways to modulate pigment production [18, 45]. In Phalaenopsis spp., PeMYB11 has been shown to activate the expression of the anthocyanin biosynthetic genes PeF3H5, PeDFR1 and PeANS3 [46]. In Dendrobium hybrid orchids, DhMYB2 has been reported to interact with DhbHLH1 to regulate the expression of DhDFR and DhANS [47]. In addition, MiMYB1 has been shown to interact with MibHLH2 and
MiWDR1 to activate the transcription of the anthocyanin biosynthetic genes MiF3’H, MiDFR, and MiANS in Matthiola incana flowers [48]. Some R2R3-MYB repressors have also been identified in plants in recent years, including PtrMYB182 and PpMYB57 from poplar, PpMYB17-20 from peach, MdHb1, MdMYB16, and MdMYB15L from apple, NtMYB2 from Chinese narcissus, and CmMYB7 from chrysanthemum [49–55]. In the present study, 26 R2R3-MYBs and 9 bHLHs were identified, and their expression profiles in the S2 stage were analyzed. The R2R3-MYBs were used to construct a phylogenetic tree with anthocyanin-related R2R3-MYBs from other plants. Notably, one R2R3-MYB (TRINITY_DN55156_c1_g2) exhibited 10.5-fold higher expression levels in ‘Yanzhi Mi’ petals than in ‘Dayuanyangjin’ petals in the S2 stage; one R2R3-MYB (TRINITY_DN59015_c3_g2) that was significantly downregulated in ‘Yanzhi Mi’ petals in the S2 stage was found to be closely related to the anthocyanin-regulated gene Tca MYB112 in cacao [39]. Further research on these candidate transcription factors is needed to confirm our hypothesis.

Conclusions

In conclusion, a combination of analytical chemistry and transcriptome analyses was performed to elucidate the molecular basis underlying pink pigmentation in the flowers of the ‘Yanzhi Mi’ mutant and white pigmentation in the flowers of WT ‘Dayuanyangjin’ plants. Seven derivatives of cyanidin, pelargonidin and peonidin were deemed to be the main contributors to pink color formation. We identified potential candidate genes encoding key enzymes in the anthocyanin biosynthetic pathway, such as CHS, CHI, F3’H, and F3’H, which are significantly differentially expressed in the pink flowers of ‘Yanzhi Mi’ and the white flowers of ‘Dayuanyangjin’ in the key stage of flower color formation, the S2 stage. These genes were the most likely candidates responsible for flower coloration in ‘Yanzhi Mi’. In particular, two R2R3-MYB transcription factors that might be involved in pink flower coloration regulation in ‘Yanzhi Mi’ were also identified. The most likely cause of color variation in the flowers of ‘Yanzhi Mi’ was proposed and discussed.

Supplementary Information

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Author contributions

JS, CL, LH and XL designed the experiments; XS, HZ, ZX handled experimental material; XS, ZX, HG and ZG were responsible for software and data processing; LH and XS writing original draft preparation; XS and ZG were responsible for review, editing and visualization. LH, HZ and CL were responsible for funding acquisition. XS and LH contributed equally to this work. All authors have read and agreed to the published version of the manuscript.

Data availability

Transcriptome raw sequence data in this study are available in SRA at NCBI with the BioSample accession number of SAMN17817099; data pertaining to the study have been included in this manuscript and supplementary materials. A pre-print of the paper exists on Research Square (https://www.researchsquare.com/article/rs-263287/v1).

Declarations

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

This manuscript does not contain any studies conducted on human or animal subjects.

Consent to publish

Not applicable.

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