



Article

Integrated Transcriptome and Metabolome to Elucidate the Mechanism of Aluminum-Induced Blue-Turning of Hydrangea Sepals

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Abstract: *Hydrangea macrophylla* is an ornamental plant with varied calyx colors. Interestingly, from red, to purple, to blue, the colors of all *Hydrangea macrophylla* are formed by unique delphinidin-3-O-glucoside and aluminum ions (Al^{3+}) and 5-O-p-coumaroylquinic acid. The sepals of 'Blue Mama' changed from pink to blue, and the contents of delphinidin-3-O-glucoside and aluminum ions increased under 3 g/L aluminum sulfate treatment. However, the mechanism of the effect of aluminum ions on the synthesis and metabolism of anthocyanins in *Hydrangea macrophylla* is still unclear. In this project, transcriptome sequencing and anthocyanin metabolome analysis were performed on the sepals of 'Blue Mama' during flower development at the bud stage (S1), discoloration stage (S2) and full-bloom stage (S3) under aluminum treatment. It was found that delphinidin, delphinidin-3-O-glucoside and delphinidin-3-O-galactoside were the main differential metabolites. The structural genes *CHS*, *F3H*, *ANS*, *DFR* and *BZI* in the anthocyanin synthesis pathway were up-regulated with the deepening in sepal color. There was no significant difference between the aluminum treatment and the non-aluminum treatment groups. However, seven transcription factors were up-regulated and expressed to regulate anthocyanin synthesis genes *CHS*, *F3H*, *BZI* and *4CL*, promoting the sepals to turn blue. The KEGG enrichment pathway analysis of differentially expressed genes showed that the glutathione metabolism and the ABC transporter pathway were closely related to anthocyanin synthesis and aluminum-ion transport. *GST* (Hma1.2p1_0158F.1_g069560.gene) may be involved in the vacuolar transport of anthocyanins. The expression of anthocyanin transporter genes *ABCC1* (Hma1.2p1_0021F.1_g014400.gene), *ABCC2* (Hma1.2p1_0491F.1_g164450.gene) and aluminum transporter gene *ALS3* (Hma1.2p1_0111F.1_g053440.gene) were significantly up-regulated in the aluminum treatment group, which may be an important reason for promoting the transport of anthocyanin and aluminum ions to vacuoles and making the sepals blue. These results preliminarily clarified the mechanism of aluminum ion in the synthesis and transport of anthocyanin in *Hydrangea macrophylla*, laying a foundation for the further study of the formation mechanism of 'blue complex' in *Hydrangea macrophylla*.

Keywords: *Hydrangea macrophylla*; aluminum; blue sepals; glutathione S-transferase; anthocyanin transport



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1. Introduction

Hydrangea macrophylla is a deciduous shrub of the Hydrangeaceae family, and it is also an aluminum-enriched plant. Its petal-like sepals are the main ornamental parts, including white, pink, blue and purple, and the color of sepals can change with the content of aluminum ions in the sepals. This phenomenon has also attracted the attention of botanists and horticulturists [1].

Flower color is an important trait of ornamental plants, and the formation of plant flower color is affected by a combination of factors, including core anthocyanin types, anthocyanin transport methods, anthocyanin accumulation and metal ions. Anthocyanin biosynthesis pathway is an important branch of plant flavonoid pathway, and its biosynthesis is regulated by structural genes and transcription factors. The structural genes include upstream structural genes *CHS*, *CHI* and *F3H*, and downstream structural genes *DFR*, *F3'H*, *F3'5'H*, *ANS* and *UFGT*. Among them, *F3'5'H* is a key enzyme gene for the synthesis of blue flower core pigment delphinidin. The expression of these structural genes is regulated by transcription factors such as MYB, bHLH and WD40, which regulate the expression of genes related to anthocyanin biosynthesis by binding to the corresponding cis-acting elements in the structural gene promoter [2]. In the cultivars of *Hydrangea macrophylla*, the same anthocyanin (delphinidin-3-O-glucoside) can make sepals appear red, purple and blue [3]. At present, the structural genes *F3H* and *DFR* in the anthocyanin synthesis pathway and the transcription factor *HymMYB2* related to the formation of blue *Hydrangea macrophylla* have been identified from *Hydrangea macrophylla* [4,5].

The transport and storage of anthocyanin to vacuole after anthocyanin synthesis is also a key process affecting plant coloring. Anthocyanin transporters play an important role in the accumulation of anthocyanin. At present, four kinds of anthocyanin transport models have been proposed, and it has been found that GST, MRP, MATE and BTL-homologous proteins may be involved in the transport of anthocyanin to vacuoles [6]. The protein structure of GSTs determines the diversity of their functions. Studies have confirmed that *GSTU* involved in flavonoid accumulation is involved in the regulation of anthocyanins. *GSTF* substrates are widely available and are involved in the accumulation of many components of flavonoids, and *GSTF12* encoded by *Petunia* (*Petunia hybrida*) *PhAn9* directly binds to anthocyanins, transporting them to the vesicles [7]. Maize (*Zea mays* L.) *Bz2* was the first GST family member found to be related to anthocyanin glycoside transport [8], and GST genes related to anthocyanin accumulation were subsequently reported in cyclamen (*Cyclamen persicum*) [9], chrysanthemums (*Chrysanthemum morifolium* Ramat.) [10], guayule (*Senecio cruentus*) [11], peonies (*Paeonia suffruticosa*) [12] and other ornamental plants. ABC transporters play a key role in plant secondary metabolite transport, exogenous toxin detoxification, lipid metabolism and plant disease resistance. Some members of the ABCC/MRP subfamily act as glutathione S-crosslinking binding pumps on the vacuolar membrane and participate in the transmembrane transport of anthocyanins. In the mutant analysis of maize and petunia, it was found that GST-deficient mutants could not accumulate anthocyanins into vacuoles. MRP/ABCC not only had a substrate preference for glutathione conjugates, but also improved its transport activity [8,13]. Similar findings suggest that *Arabidopsis* *AtMRP1* and *AtMRP2* [14,15], maize *ZmMRP3* [16], rice (*Oryza sativa* L.) *OsMRP15* [17] and grape (*Vitis vinifera* L.) *ABCC1* are associated with transmembrane transport of anthocyanin glycosides [18].

Studies have confirmed that in acidic soil, *Hydrangea macrophylla* absorbs and transports aluminum ions to the sepals, and the content of Al^{3+} in the sepals of blue *Hydrangea macrophylla* is about 40 times that of red sepals [19]. The 'blue complex' in the blue sepals is composed of delphinidin-3-O-glucoside, aluminum ions and 5-O-acylquinic acid in a ratio of 1:1:1 [20–22]. As an aluminum hyperaccumulator, *Hydrangea macrophylla* has a natural tolerance to aluminum ions. The aluminum transporter genes *HmPALT* and *HmVALT* on the plasma membrane and vacuole membrane have been cloned from the sepals of *Hydrangea macrophylla* [23]. Aluminum treatment not only activates the activity of transporters, but also promotes the increase in anthocyanin content in the sepals and makes the sepals' color blue; the *ABCC1* gene of hydrangea was also up-regulated by aluminum, which promoted the accumulation of Al^{3+} in the sepals [24–26]. At present, many advances have been made in understanding the chemical mechanism of sepal color formation in *Hydrangea macrophylla*, including the structure of the blue complex, the transporters involved in the accumulation of aluminum ions and the distribution of aluminum ions in the sepals. How-

ever, the molecular mechanism of aluminum-induced sepal color formation in *Hydrangea macrophylla* remains to be further studied.

We conducted this experiment in order to explore the molecular mechanism and metabolic pathway of sepal color change caused by aluminum treatment and to determine the key genes regulating color formation. In this study, transcriptome sequencing and metabolite analysis were performed on the sepals of Al-treated *H. macrophylla* 'Blue Mama' at the bud stage (S1), discoloration stage (S2) and full-bloom stage (S3). The changes in metabolites during the process of sepals turning blue were studied, the differentially expressed genes and transcription factors related to anthocyanin synthesis and transport were identified, and the related transcriptional regulatory network was constructed to reveal the differential regulation of genes involved in anthocyanin accumulation. This finding is helpful to further elucidate the regulatory mechanism of aluminum-induced anthocyanin accumulation to form blue flowers.

2. Materials and Methods

2.1. Plant Materials and Experimental Treatment

Three-year-old hydrangea plants of the 'Blue Mama' variety, uniform in the growth stage and size, were selected and planted in five-gallon pots filled with a mixed substrate (vermiculite/perlite/peat/garden soil = 2:2:3:3). These pots were placed in the flower base of the College of Horticulture, Hunan Agricultural University, for uniform maintenance and management. The first aluminum treatment was started at the budding stage in early March 2022. The concentrations of $\text{Al}_2(\text{SO}_4)_3$ (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) were 1, 3 and 5 g/L, respectively, and water was used as the control. The treatment was performed once every 10 days during the growth and development process, with 3 pots for each treatment and 3 biological replicates. According to the characteristics of sepals at different stages of flower development, they were divided into S1 stage (bud stage), S2 stage (discoloration stage) and S3 stage (full-bloom stage). Three inflorescences were randomly selected from each treatment, and three sepals were taken from each inflorescence. This process was repeated three times, and the samples were stored at -80°C .

2.2. Flower Color Determination

The Royal Horticultural Society Color Chart (RHSCC) and colorimeter (3nh-YS30, Shenzhen Sanenshi Technology Co., Ltd., Shenzhen, China) were used to determine the color (brightness L^* value, redness a^* value, blueness b^* value) of the middle part of the calyx of *Hydrangea macrophylla*. Each calyx was measured three times, and the average value was taken [27].

2.3. Determination of Metal ion Content

At the S3 stage, the roots, stems, leaves and fresh sepals of the plants were taken, washed and dried in an oven to constant weight. Then, the samples were ground into powder and passed through a 100-mesh sieve. A 0.1 g portion of the ground sample was weighed, and 5 mL of nitric acid was added for digestion. The mixture was diluted to 50 mL with distilled water and then diluted 10 times. The content of Al^{3+} was determined by ICP-MS (Agilent 7900 ICP-MS, Agilent Technologies, Santa Clara, CA, USA) [28]. Each treatment group was set up with 3 replicates.

2.4. Extraction and Determination of Anthocyanin

The fresh sepals were placed in a mortar and quickly ground to a fine powder with liquid nitrogen. The 250 mg powder was weighed in a centrifuge tube. After the liquid nitrogen was volatilized, 1 mL anthocyanin extract (Vmethanol/Vwater/Vformic acid = 70:27:3) was added. Then, it was placed on a shaker and shaken well. The centrifuge tube was wrapped completely with tin paper and placed in a refrigerator at 4°C for 12 h. After the extraction was completed, it was shaken once again. Then, it was transferred to a centrifuge (5424R, Eppendorf AG, Hamburg, DE) at 4°C and centrifuged at 10,000 rpm/min for 5 min.

The supernatant was taken, filtered through a 0.45 µm filter membrane, and the filtrate was stored in an injection bottle. It was then detected and analyzed by high performance liquid chromatography (CBM-20A, Shimadzu, Kyoto, Japan). The chromatographic column was Welch[®] C18 (4.6 mm × 250 mm, 5 µm). The mobile phase A was 5% formic acid aqueous solution, and the mobile phase B was 5% formic acid acetonitrile. The flow rate was 1.0 mL/min. Elution gradient: 0 min, 5% B; 8~13 min, 13% B; 13~20 min, 17% B; 20~23 min, 17% B; 23~30 min, 20% B; 30~40 min, 20% B; 40~40.1 min, 5% B; 40.1~50 min, 5% B. The detection wavelength was 520 nm. The external standard method was used to quantify the sample. The standard was delphinidin-3-O-glucoside, and the standard curve was $y = 40,922x - 1166.5$ ($R^2 = 1$).

2.5. Metabolite Extraction and Analysis

Metabolome analysis was performed using 18 sepal samples (sepals at three stages of flower development in the CK group and the 3 g/L aluminum treatment group, three biological replicates). Weigh 20 mg of freeze-dried and ground calyx samples in a 2 mL centrifuge tube, add 1 mL of methanol: aqueous solution (7:3, *v/v*, containing 0.1% formic acid) vortex for 30 s, homogenize at 45 Hz for 15 min and ultrasound for 30 min under ice water bath conditions. The samples were centrifuged at 4 °C and 12,000 r/min for 15 min, and 200 µL of the supernatant was filtered through a 0.22 µm filter membrane and analyzed by the UHPLC-MS/MS system.

UHPLC conditions: the UHPLC separation was carried out using a Waters ACQUITY I-Class (Waters Corporation, Milford, MA, USA), equipped with an ACQUITY UPLC HSS T3 (100 mm × 2.1 mm, 1.8 µm, Waters). The mobile phase A was 0.1% formic acid in water, and the mobile phase B was 0.1% formic acid in acetonitrile. Elution gradient: 0 min, 5% B; 1 min, 5% B; 4 min, 30% B; 9 min, 60% B; 12 min, 80% B; 14 min, 5% B. The column temperature was set to 35 °C. The auto-sampler temperature was set to 10 °C, and the injection volume was 2 µL.

Mass spectrometer conditions: a SCIEX 6500 QTRAP + triple quadrupole mass spectrometer (SCIEX, Hermosa Beach, CA, USA) equipped with IonDrive Turbo V ESI ion source was used for mass spectrometry analysis in multiple reaction monitoring (MRM) mode. Multi-Response Monitoring (MRM) was developed by biomarker biotechnology (Beijing, China). The ion-source parameters were as follows: curtain gas = 35 psi, IonSpray voltage = +5500 V, −4500 V, temperature = 550 °C, ion-source gas 1 = 50 psi, ion-source gas 2 = 55 psi.

In this study, qualitative and quantitative analysis of anthocyanin metabolites was carried out based on the UHPLC-MS/MS platform, combined with standard substances. The information on standard substances, standard curves and method detection limits is shown in Table S1. The screening criteria for differential metabolites were fold change ≥ 1 , VIP ≥ 0 and $p < 0.05$.

2.6. Transcriptome Analysis

As with the samples used for metabolomics analysis, 18 sepal samples were used for transcriptomics analysis. Total RNA was extracted from each sepal samples using RNAPrep Pure Plant Kit reagent (Tiangen, Beijing, China). RNA quality and integrity were detected using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The cDNA library was generated using the NEBNext[®] Ultra[™] RNA non-strand-specific library preparation kit (NEB, Ipswich, MA, USA). After the cDNA library quality was evaluated by the Agilent Bioanalyzer 2100 system, sequencing was performed on the Illumina high-throughput sequencing platform. The original sequence data containing adaptors and low-quality reads ($N\% > 10\%$, $Q \leq 10$ bases $> 50\%$) were removed to obtain high-quality clean data. The clean reads were aligned to the *Hydrangea macrophylla* reference genome (reference genome version information: *Hydrangea macrophylla*.HMA_r1.2_1.genome.fa)

using HISAT2 software. After the alignment analysis was completed, StringTie software (v2.2.0) was used for assembly, quantification and standardization using FPKM.

DESeq2 was used to identify differentially expressed genes (DEGs) between different samples (fold change ≥ 1.5 , FDR < 0.05). STEM v1.3.13 was used to analyze the temporal expression trend of DEGs, and then TBtools software (version 2.086) was used to perform KEGG (<https://www.genome.jp/kegg/>, accessed on 16 October 2023) pathway enrichment analysis on DEGs, within the significant enrichment trend ($p < 0.05$).

2.7. qRT-PCR

qRT-PCR was performed using the ChamQ Universal SYBR qPCR Master Mix reagent (Vazyme, Nanjing, China) and the Bio-Rad CFX96 RT-PCR system (Bio-Rad, Hercules, CA, USA). Each sample was analyzed in triplicate. Specific primers were designed using Primer Premier 5.0 (Table S2). The relative gene expression level was calculated by the $2^{-\Delta\Delta CT}$ method [29].

2.8. Data Analysis

Excel 2019 and SPSS 20.0 were used for data statistics, as well as one-way analysis of variance (ANOVA); $p < 0.05$ was statistically significant. Correlation analysis was performed using the Maiwei Cloud platform (<https://cloud.metware.cn/>, accessed on 20 November 2023). Diagrams were produced using Graphpad Prism 8.0, Adobe Photoshop 2020 and Cytoscape v3.7.1.

3. Results

3.1. The Changes in Calyx Color and Metal Ion Content under Different Concentrations of Aluminum Treatment

Figure 1A,B and Table S3 show that with the development of flowers, the calyx began to color from green (S1) and completely colored at the S3 stage. Aluminum treatment made the sepals color turn blue. With the increase in the aluminum treatment concentration, the sepals were light purple (P-V N82D), blue (B101C) and purple blue (V-B98C), and the content of delphinidin-3-O-glucoside also increased. The content of aluminum ions in each part of the plant was measured during the full-bloom stage. It was found that the aluminum treatment promoted the upward transport of aluminum ions in the plant and accumulated them in the sepals and leaves. This trend was consistent with the change trend in the delphinidin-3-O-glucoside content. However, when the aluminum treatment concentration was 5 g/L, a large amount of aluminum ions accumulated in the roots, which may cause aluminum toxicity (Figure 1C). It was concluded that aluminum treatment promoted the accumulation of anthocyanins and aluminum ions in the sepals. When the concentration of aluminum sulfate was 3 g/L $Al_2(SO_4)_3$, ‘Blue Mama’ formed blue sepals.

3.2. Metabolome Analysis of Anthocyanins

In order to understand the changes in the anthocyanin composition and content during the sepal coloration of *Hydrangea macrophylla*, the changes in anthocyanin metabolites during three developmental stages were detected by the UHPLC-MS/MS method. A total of 53 anthocyanin metabolites were detected in the samples, including 13 delphinidins, 13 cyanidins, 5 petunidins, 5 peonidins, 5 pelargonidins, 3 malvidins and others (Figure 2A, Table S4). The sepals of the aluminum-free treatment group changed from green to pink. Through the analysis of differential metabolites during development, it was found that the number of differential metabolites up-regulated and down-regulated in CK-S1 vs. CK-S2, CK-S1 vs. CK-S3 and CK-S2 vs. CK-S3 were 18 and 7, 17 and 9, 12 and 8, respectively (Figure 2B). Further analysis showed that delphinidin, delphinidin-3-O-glucoside, delphinidin-3-O-galactoside and naringenin-7-O-glucoside were the main anthocyanin metabolites in the pink sepals. The contents of these four metabolites continued to increase during sepal coloration and peaked at the S3 stage.

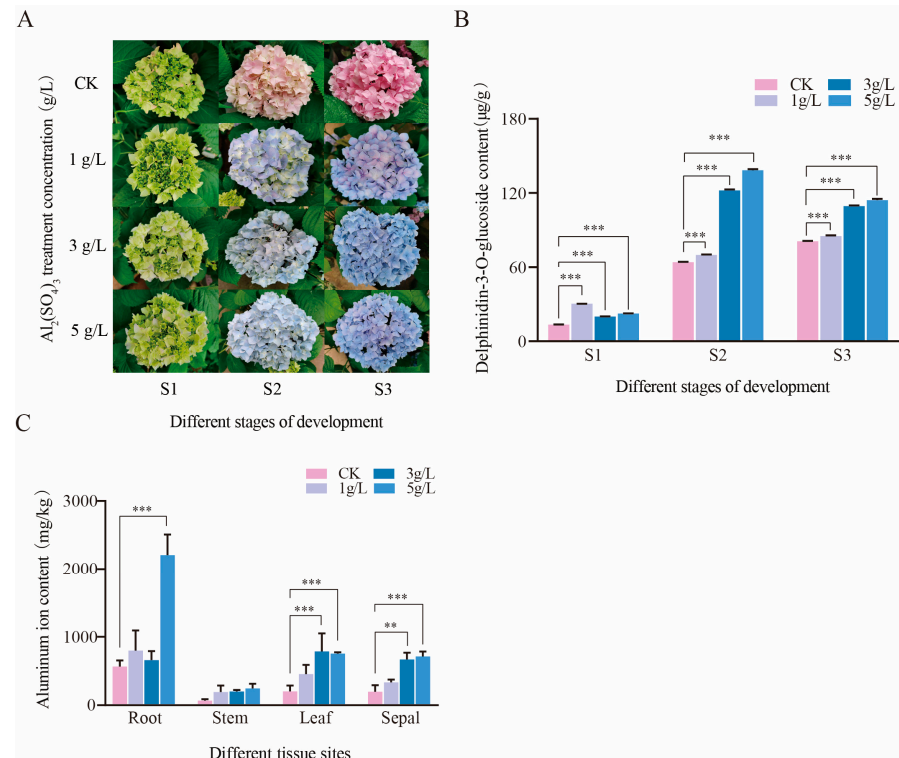


Figure 1. Changes in flower color and aluminum-ion content of ‘Blue Mama’. (A) Changes in flower color during the development of ‘Blue Mama’ under different concentrations of aluminum treatment. (B) Changes in the content of delphinidin-3-O-glucoside in sepals under different concentrations of aluminum treatment. (C) Aluminum-ion content in root, stem, leaf and sepals of plants at S3 stage. **, $p < 0.01$; ***, $p < 0.001$.

The sepals of the 3 g/L $\text{Al}_2(\text{SO}_4)_3$ treatment group changed from green to blue. Through the analysis of differential metabolites during development, it was found that in Al-S1 vs. Al-S2, Al-S1 vs. Al-S3 and Al-S2 vs. Al-S3, the number of differential metabolites up-regulated and down-regulated were 19 and 10, 17 and 10, 12 and 14, respectively (Figure 2B). Delphinidin, delphinidin-3-O-glucoside, delphinidin-3-O-galactoside and naringenin-7-O-glucoside were the main metabolites in the blue sepals. These results are the same as the results of the aluminum-free treatment group. However, the content of metabolites increased significantly from the S1 to the S2 stage and reached the peak in the S2 stage. Therefore, the S2 stage was considered to be the key period for the color of blue *Hydrangea macrophylla*.

Compared with the aluminum-free treatment group, the number of differential metabolites up-regulated and down-regulated in the three developmental stages were 5 and 6, 10 and 6, 8 and 9, respectively (Figure 2B). After aluminum treatment, the sepals were blue at the S2 stage, and the contents of delphinidin, delphinidin-3-O-galactoside and delphinidin-3-O-glucoside in the sepals were significantly higher than those in the aluminum-free treatment group (Figure 2C). Therefore, it is speculated that the significant increase in the content of delphinidin and its derivatives induced by aluminum in the S2 stage is the key period for the sepals to turn blue.

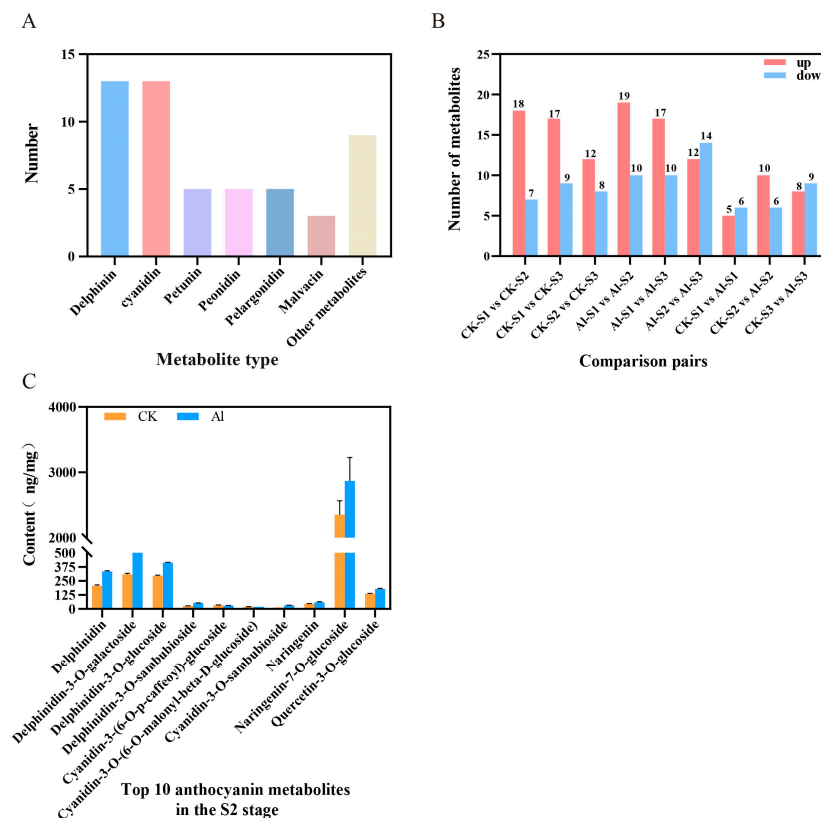


Figure 2. Statistical analysis of anthocyanin metabolites. (A) The type and quantity of metabolites identified. (B) The number of differential metabolites in different comparison groups. (C) Comparative analysis of the top 10 anthocyanin metabolites in terms of content in the S2 stage.

3.3. Calyx Transcriptome Analysis Data Statistics

Transcriptome sequencing was performed on the sepals of three stages of flower development of 'Blue Mama' treated with 3 g/L aluminum sulfate solution, and a total of 117.50 Gb clean data were obtained with water treatment as the control. The results of the PCA showed that the biological repeatability was good, and there was a strong correlation between the samples in each group ($R^2 > 0.82$) (Figure S1). Therefore, the transcriptome sequencing data is reliable and can be used for subsequent analysis. With fold change ≥ 1.5 and FDR < 0.05 as the screening conditions for differential genes (Table S5), it can be seen from Figure 3A that there are a large number of differential genes between the three stages of flower development, among which the number of differential genes in S1 and S3 stages is the largest. Compared with the control group, the number of differential genes in the S2 stage was the highest. From the Venn diagram of differential genes within and between groups, it can be seen that the number of differential genes unique to the S2 stage is the most, which is 22.8 times that of the S1 stage and 2.4 times that of the S3 stage (Figure 3B). The above results show that the period from S1 to S2 was the key period for sepal coloration of *Hydrangea macrophylla*.

3.4. Changes in Gene Expression Profiles in Different Treatment Groups

In order to further understand the relevant biological processes, the co-expression profiles of the differential gene sets of the aluminum-free treatment group and the aluminum treatment group at the three flower development stages were analyzed. The results showed that 11,988 and 12,630 differentially expressed genes in the two treatment groups were classified into eight different expression patterns (Figures S2 and S3), and there were three significant temporal expression patterns in both groups. Subsequently, KEGG enrichment analysis was performed on genes in significant expression profiles ($p < 0.05$). Profile7 of the two treatment groups contained genes that were positively regulated throughout the time

course, and some of them were enriched in plant circadian rhythms, plant hormone signal transduction, glutathione metabolism, starch and sugar metabolism, phenylpropanoid biosynthesis pathway (Figure 4). In addition, in the aluminum treatment group, the genes involved in peroxisome metabolism were also specifically enriched in Profile7 (Figure 4B). These results suggest that these biological pathways have potentially important effects on the calyx coloration of *Hydrangea macrophylla*.

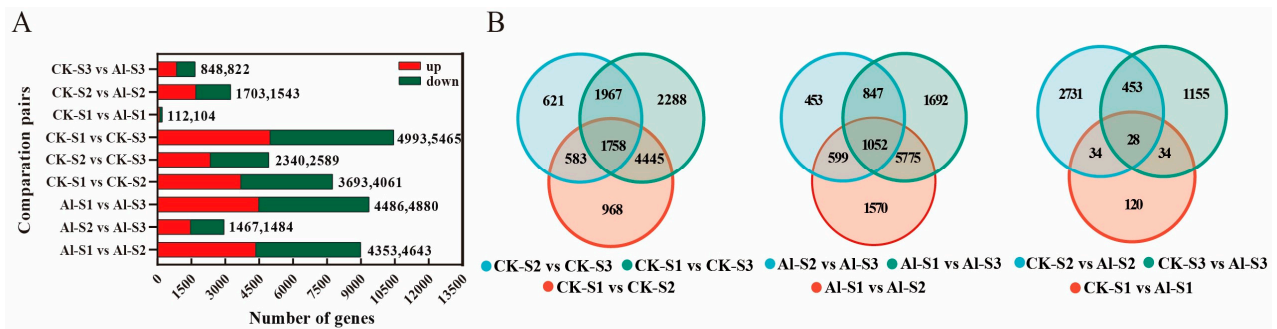


Figure 3. Sequencing results statistics. (A) Statistical analysis of differential genes between groups. (B) The differential gene Venn diagram within and between groups.

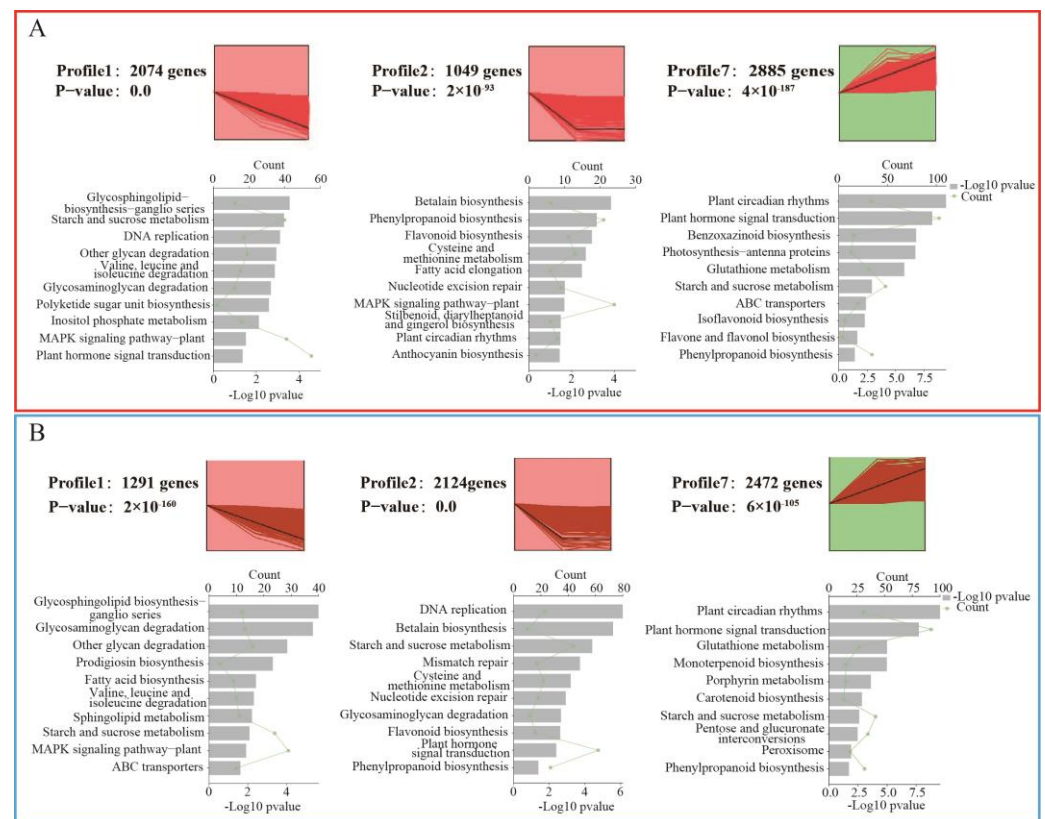


Figure 4. The expression profiles of significantly enriched differential genes and their KEGG pathway enrichment analysis. The above profiles significantly represent enriched expression patterns of differential genes, with the broken lines indicating the trend in gene expression across the three stages of flower development ($p < 0.05$). Below each cluster, the top 10 most significantly enriched KEGG pathways are represented by a histogram based on the adjusted p -value, and the number of genes in each pathway is represented by a line chart. (A,B) are the CK group and the Al treatment group, respectively.

3.5. Association Analysis of Differential Genes and Metabolites in Anthocyanin Biosynthesis

The anthocyanin biosynthesis pathway is an important metabolic branch of the flavonoid pathway, which is responsible for the production of anthocyanins in different plant tissues. Phenylalanine is a precursor for the biosynthesis of flavonoids. It is catalyzed by phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumarate CoA ligase (4CL). During the development of *Hydrangea macrophylla*, 9 *PAL*, 3 *C4H* and 7 *4CL* genes were differentially expressed. However, only 1 *4CL* (Hma1.2p1_1457F.1_g284660.gene) gene was significantly up-regulated, and the expression level of the aluminum treatment group was higher than that of the control group.

By analyzing the differentially expressed genes in the anthocyanin metabolic pathway, the results showed that during the development of *H. macrophylla* flowers, as the sepals color changed from green to pink and blue, that is, from the S1 to the S3 stage, the *CHS* (Hma1.2p1_0691F.1_g204240.gene), *F3H* (Hma1.2p1_0956F.1_g241310.gene), *ANS* (Hma1.2p1_0371F.1_g138300.gene), *DFR* (Hma1.2p1_0789F.1_g218150.gene), *BZI* (Hma1.2p1_0498F.1_g166700.gene) genes were significantly up-regulated in the two treatment groups (Figure 5A). The Pearson correlation coefficient method was used to calculate the correlation between differential genes and major metabolites in the anthocyanin biosynthesis metabolism, and a total of seven structural genes for anthocyanin biosynthesis and five differential metabolites were identified as having a correlation with each other (Figure 5B, Table S6). Among them, delphinidin, delphinidin-3-O-galactoside and delphinidin-3-O-glucoside were positively correlated with *CHS* (Hma1.2p1_0691F.1_g204240.gene) and *F3H* (Hma1.2p1_0956F.1_g241310.gene), indicating that the differential expression of the *CHS* and *F3H* structural genes played an important role in the pink or blue sepals of *Hydrangea macrophylla* (Table S6). However, compared with the non-aluminum treatment group, the expression of the *CHS* and *F3H* genes in the sepals of *H. macrophylla* was not significantly up-regulated after aluminum treatment. Therefore, it is speculated that the up-regulation of the anthocyanin biosynthesis genes induced by aluminum may not be the main reason for the blue sepals.

3.6. Expression Analysis of Transcription Factors Related to Anthocyanin Biosynthesis

A total of 69 transcription factor families were identified during the floral development of *Hydrangea macrophylla*, of which the NAC and bHLH genes were the most abundant, with 138 and 131 members, respectively, followed by MYB, C2H2, AP2/ERF-ERF and other gene families (Figure 6A). Based on the differentially expressed genes FPKM > 5 in at least one sample, 17 *NAC* genes, 23 *bHLH* genes and 21 *MYB* genes were screened. In the aluminum treatment group, the expression of 11 *NAC* genes, 8 *bHLH* genes and 7 *MYB* genes were significantly up-regulated during the key period (S2) of sepal color from green to blue. In addition, eight *bHLH* genes and six *MYB* genes were significantly down-regulated (Figure 6B–D).

In order to reveal the potential biological regulatory network of anthocyanin synthesis-related gene expression, we constructed co-expression networks of differentially expressed transcription factors with anthocyanin synthesis structural genes and found that 9 *NAC*, 5 *bHLH*, and 8 *MYB* transcription factors were correlated with the anthocyanin synthesis structural genes ($p < 0.05$). Among these, *NAC* (Hma1.2p1_1079F.1_g254920.gene, Hma1.2p1_1124F.1_g259533.gene) and *MYB* (Hma1.2p1_0457F.1_g156980.gene, Hma1.2p1_0653F.1_g196210.gene) were highly expressed in the S2 stage of the aluminum-treated group and correlated positively with the anthocyanin synthesis structural genes *4CL* (Hma1.2p1_1457F.1_g284660.gene) and *F3H* (Hma1.2p1_0956F.1_g241310.gene) (Figure 6E,G). As shown in Figure 6F,G, *bHLH* (Hma1.2p1_0523F.1_g172150.gene, Hma1.2p1_0301F.1_g118230.gene) and *MYB* (Hma1.2p1_0061F.1_g033690.gene) were highly expressed in the aluminum-treated group during the S2 stage and were associated with *BZI* (Hma1.2p1_0498F.1_g166700.gene) and *CHS* (Hma1.2p1_0691F.1_g204240.gene), which were positively correlated (Table S6). Therefore, it was speculated that aluminum induced the up-regulation of these seven transcription factors to regulate the anthocyanin synthesis

genes *CHS*, *F3H*, *BZI* and *4CL* and promoted the sepals to turn blue. In addition, the interaction between NAC and MYB and the interaction between bHLH and MYB are worthy of further study.

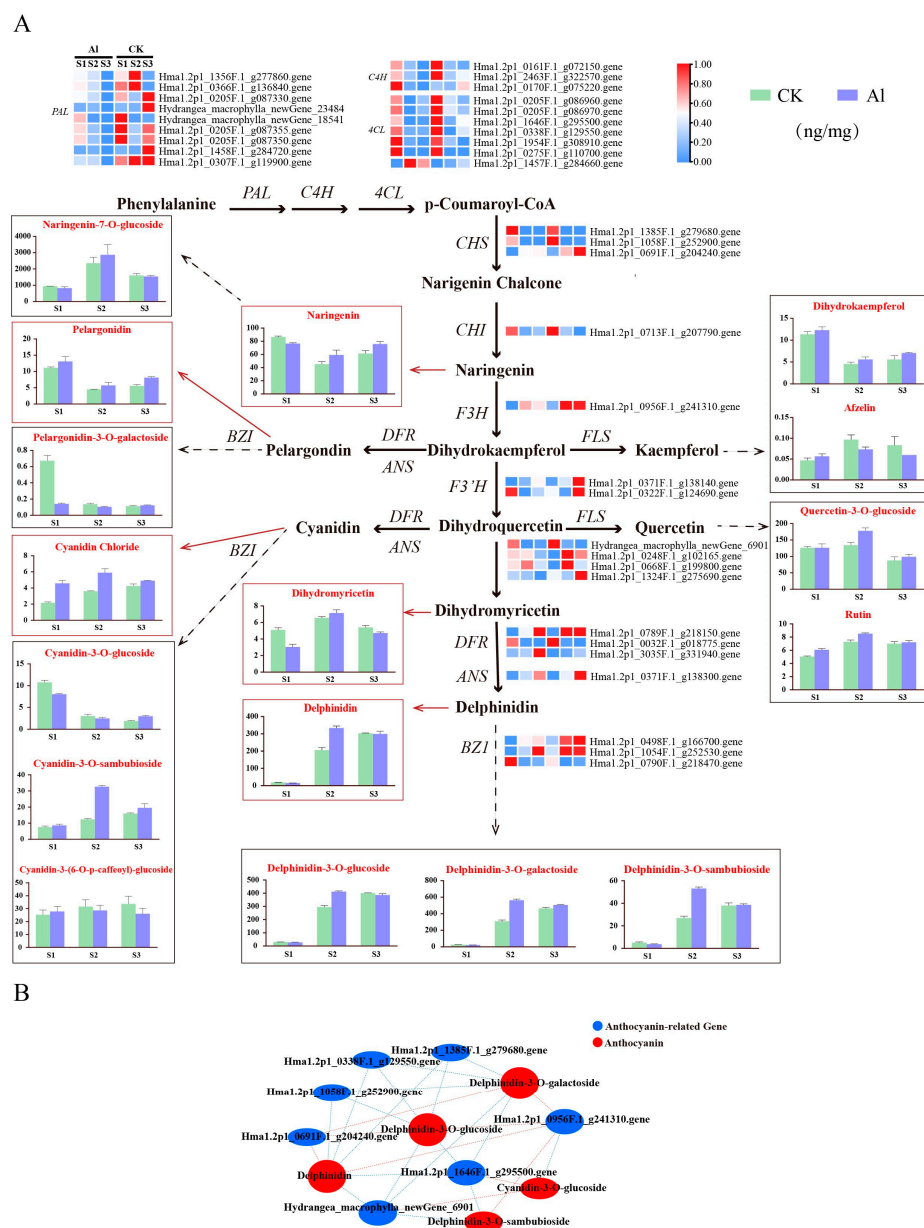


Figure 5. Comparative analysis of gene expression and metabolites in anthocyanin biosynthesis pathway at different stages of flower development. **(A)** Comparative analysis of structural gene expression and metabolite content. **(B)** Correlation analysis between structural genes and metabolites. Only Pearson correlation coefficient $|PCC| \geq 0.90$, $p < 0.05$. Red dashed line, positive correlation between gene and metabolite; blue dashed line, negative correlation between gene and metabolite.

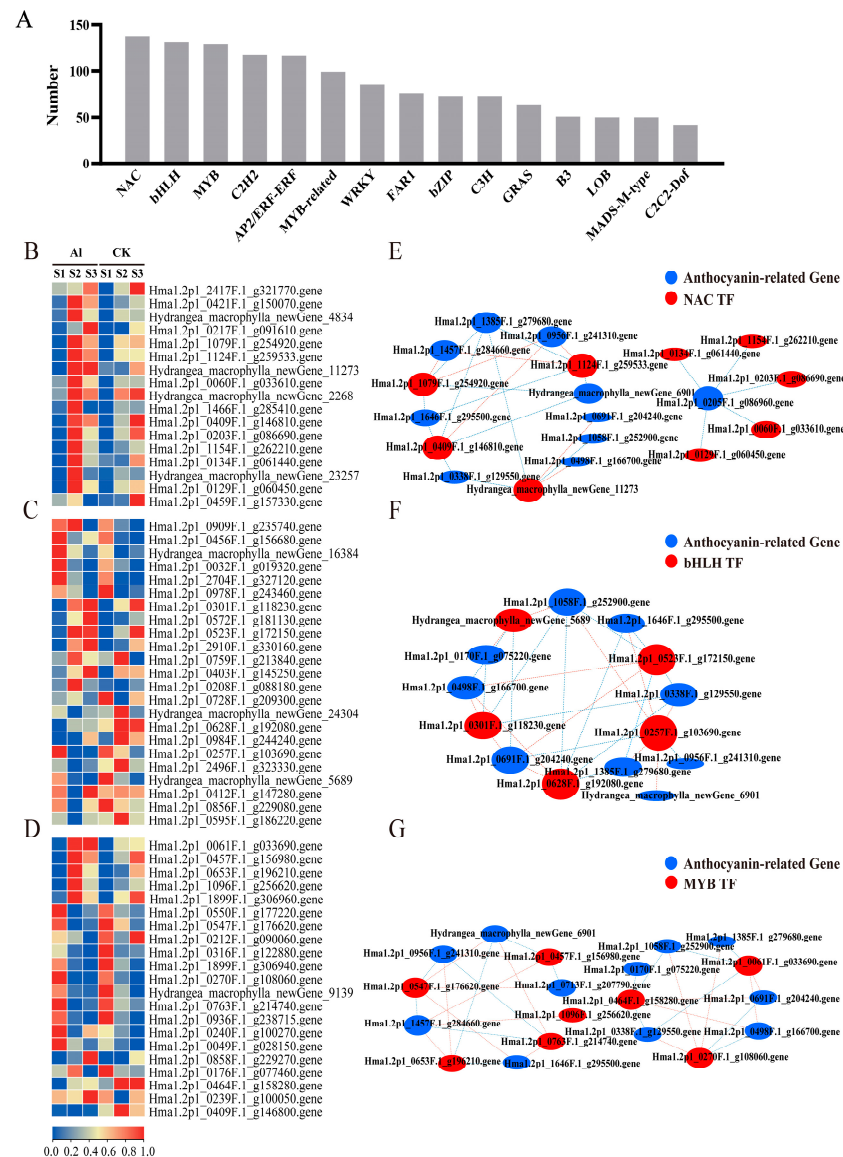


Figure 6. Transcription factor analysis. (A) The number of top 15 transcription factor families identified. (B) Heatmap of NAC transcription factor. (C) Heatmap of bHLH transcription factor. (D) Heatmap of MYB transcription factor. (E) Co-expression map of NAC and anthocyanin synthesis structural genes. (F) Co-expression map of bHLH and anthocyanin synthesis structural genes. (G) Co-expression map of MYB and anthocyanin synthesis structural genes. Only Pearson correlation coefficient $|PCC| \geq 0.90$, $p < 0.05$. Red dashed line, positive correlation between genes; blue dashed line, negative correlation between genes.

3.7. Anthocyanin Transporter Gene Expression Analysis

The process of anthocyanin transport and storage to vacuoles after synthesis also directly affects the coloration of plants. It can be seen from Figure 4 that the up-regulated differential genes (Profile7) were significantly enriched in the glutathione metabolic pathway and the ABC transporter pathway during the process of sepals changing from green to pink or blue. By analyzing the differentially expressed genes in the glutathione metabolic pathway, 20 *GST* genes, 2 *GSH* genes and 2 *GSH-Px* genes were identified, of which the At-GSTF12 homologous gene Hma1.2p1_0158F.1_g069560.gene was significantly up-regulated (Figure 7). Therefore, it is believed that this gene may be involved in the vacuolar transport of anthocyanin.

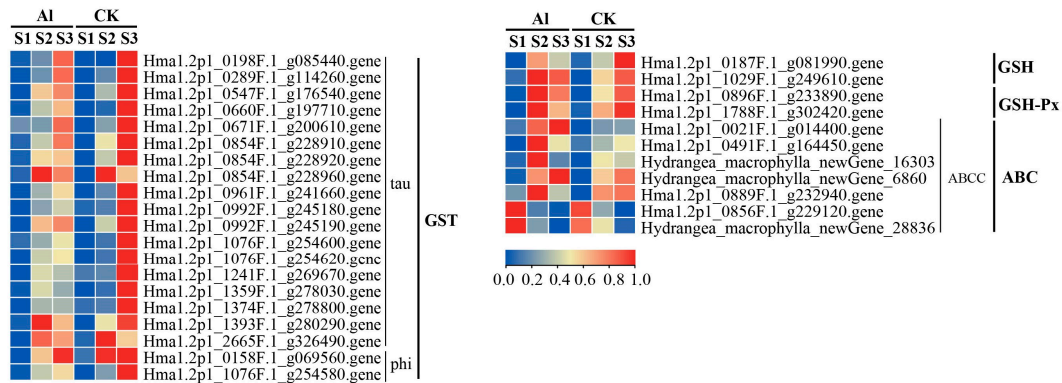


Figure 7. Expression analysis of anthocyanin transporter gene.

In the subfamily of ABC transporters, the multidrug resistance-associated protein (MRP/ABCC) is involved in the transmembrane transport of glutathione anthocyanins. It can be seen from Figure 7 that seven ABCC transporter genes were differentially expressed during the development of *Hydrangea macrophylla*, and the expression levels of four genes were up-regulated, as the color of sepals changed to pink or blue. In the aluminum treatment group, ABCC1 (Hma1.2p1_0021F.1_g014400.gene) and ABCC2 (Hma1.2p1_0491F.1_g164450.gene) were highly expressed in the S2 and S3 periods and were significantly higher than those in the aluminum-free treatment group. Therefore, it is believed that aluminum induces the high expression of ABCC1 and ABCC2 transporter genes in *Hydrangea macrophylla*, which are involved in the vacuolar transport of anthocyanins and contribute to making its calyx blue.

3.8. Expression Analysis of Aluminum Transport-Related Genes

Metal ions are one of the important factors affecting the color change in anthocyanins. The formation of complexes between metal ions and anthocyanins in vacuoles will change the color of anthocyanins, thus affecting the flower color of plants. For example, the combination of aluminum ions and anthocyanins will make the anthocyanins blue [30]. At the same time, anthocyanins can also compartmentalize metal ions and improve the tolerance of plants to metals. The main way is to reduce metal toxicity by catalyzing the binding of GSH to metals by GST [31]. It can be seen from Figure 7 that seven GST (tau) genes and one GSH-Px gene in the aluminum treatment group were significantly up-regulated at the S2 stage compared to the non-aluminum treatment group. Among them, Hma1.2p1_0992F.1_g245190.gene and Hma1.2p1_0961F.1_g241660.gene are homologous to *AtGSTU7* and *ZmGST23*, respectively, both of which are known to respond to environmental stress. Therefore, it is believed that these two genes may be involved in the vacuolar transport of aluminum ions.

A large number of studies have shown that multiple members of the ABCB, ABCG and ABCI subfamilies are involved in many key physiological processes in response to environmental stress [32]. As shown in Figure 8, four ABCB, nine ABCG and four ABCI genes were differentially expressed in the aluminum treatment group. Among them, the ABCB1 (Hma1.2p1_0608F.1_g188210.gene), ABCG2 (Hydrangea_macrophylla_newGene_12170), ABCG36 (Hma1.2p1_0430F.1_g151450.gene) gene expression levels were significantly higher than those in the aluminum-free treatment group. At the same time, four half-molecule ABC transporters, ALS3, were identified, and the expression of ALS3 (Hma1.2p1_0111F.1_g053440.gene) was significantly up-regulated in the aluminum treatment group. The above results indicate that these four ABC transporter genes may also be involved in the vacuolar transport of aluminum ions.

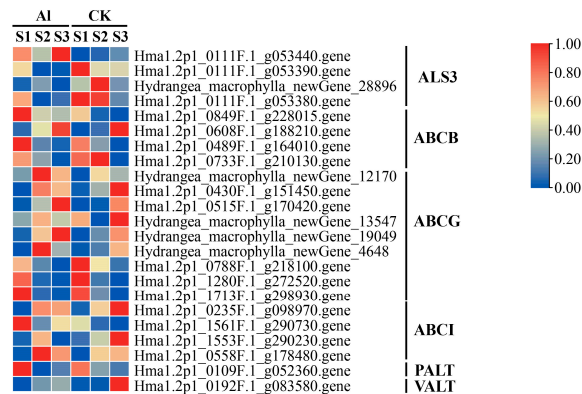


Figure 8. Expression analysis of aluminum transport-related genes.

3.9. Real-Time PCR Validation

To verify the accuracy of the transcriptome sequencing results, genes related to anthocyanin biosynthesis and transport were selected for real-time fluorescence quantification. The results showed that the selected 12 differentially expressed genes showed a similar trend to the transcriptome sequencing results, indicating that the transcriptome data of this study were reliable (Figure 9).

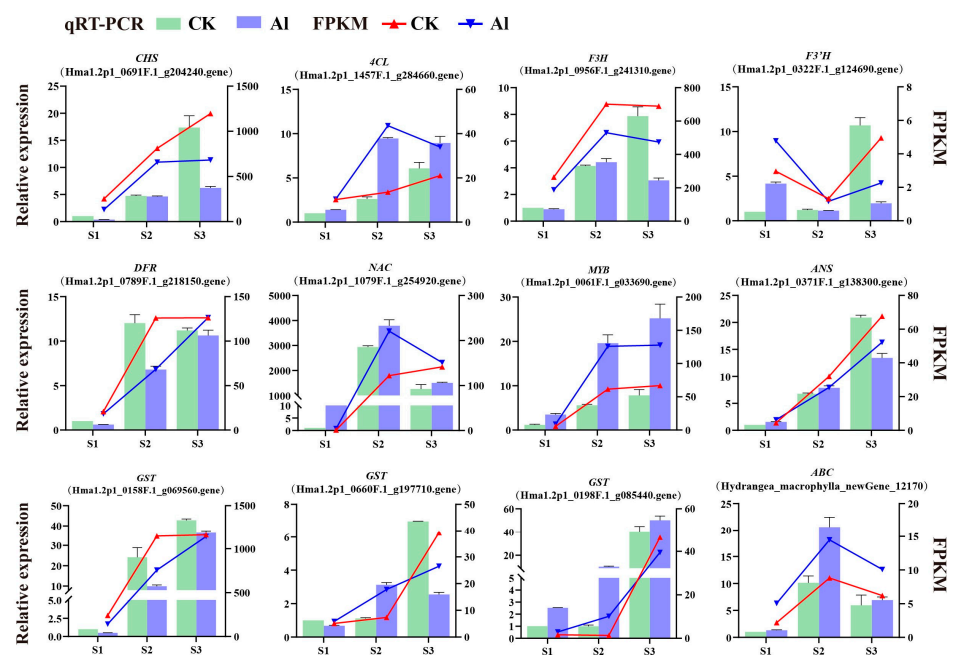


Figure 9. Validation and expression analysis of selected genes using qRT-PCR.

4. Discussion

The 'blue complex' in the calyx of *Hydrangea macrophylla* is composed of delphinidin-3-O-glucoside, 5-O-caffeoylquinic acid and Al^{3+} in a 1:1:1 ratio, in an aqueous solution with a pH value of about 4.0. Studies have shown that after soil application of aluminum sulfate, it not only reduces the pH of the soil, promotes the absorption of Al^{3+} by the roots of *Hydrangea macrophylla* and increases the content of Al^{3+} in the calyx, but also promotes the increase in the content of delphinidin-3-O-glucoside, making the calyx change from red to blue [33]. After applying different concentrations of aluminum solution, the calyx color of *Hydrangea macrophylla* changed from pink to purple and blue-purple, and the content of Al^{3+} in the calyx also increased from 2.24 ug/g FW to 5.12 ug/g FW and 11.83 ug/g FW [24]. In this study, with the increase in the aluminum treatment concentration, the color of the calyx of 'Blue Mama' changed from pink to purple, blue and blue-purple. The

content of delphinidin-3-O-glucoside and Al^{3+} in the calyces of the aluminum treatment group increased significantly, and the calyces of the 3 g/L aluminum treatment group were blue. However, the roots of the 5 g/L aluminum treatment group accumulated a large amount of aluminum ions, and the content of aluminum ions was significantly higher than in the other parts. Therefore, the appropriate concentration of aluminum treatment was very important for the blue coloration of *Hydrangea macrophylla*.

Plants mitigate aluminum toxicity through uptake, transport and compartmentalization of Al^{3+} by transporter proteins. In Arabidopsis, *AtALS3* is an aluminum transporter protein that redistributes Al^{3+} outside of aluminum-sensitive tissues [34]; in buckwheat, *FeALS1.1* and *FeALS1.2* are involved in the internal detoxification of aluminum in roots and leaves by sequestering Al^{3+} into vesicles, respectively [35]. Rice *OsALS3* encodes a semi molecular ABC transporter protein that is induced by aluminum and is not induced by other metals or a low pH [36]. In this study, the aluminum transporter protein gene *ALS3* (Hma1.2p1_0111F.1_g053440.gene) was screened. It not only up-regulated its expression with the deepening of the calyx color, but its expression was also significantly higher in the aluminum-treated group than in the control group, which suggests that *ALS3* is involved in aluminum-ion transport and blue calyx formation in *Hydrangea macrophylla*.

The main coloring substance in plants is anthocyanin, and phenylalanine is the starting material of anthocyanin metabolic pathway, which is catalyzed by PAL, C4H and 4CL [37]. The upstream structural gene *CHS* is the core enzyme of the anthocyanin synthesis pathway, and its loss of activity will lead to the loss of anthocyanin and other flavonoids, resulting in a white-flower mutant [38]. The competition of *F3H*, *F3'H* and *F3'5'H* leads to the production of different anthocyanin metabolic branch pathways. The downstream *BZI* is the key enzyme in the last step of anthocyanin synthesis, which catalyzes anthocyanin into stable anthocyanin [39]. In this study, with the deepening of calyx color, five *PAL*, three *C4H* and six *4CL* genes in the upstream structural genes of anthocyanin synthesis were highly expressed in the S1 stage, and the expression level was significantly higher than that in the S2 and S3 stages. The phenomenon of early high expression of upstream genes may provide a prerequisite for anthocyanin synthesis, which is similar to the results of Rahmati et al. [40]. However, the expression level of *4CL* (Hma1.2p1_1457F.1_g284660.gene) increased with the deepening of the calyx color, and was significantly higher in the aluminum treatment group than in the control group. The gene expression may be related to aluminum induction. The expression levels of upstream and downstream structural genes *CHS*, *F3H*, *DFR*, *ANS* and *BZI* in the anthocyanin synthesis pathway of *Hydrangea macrophylla* were up-regulated with the deepening of color, and the expression level in the aluminum treatment group was lower than that in the control group. This finding is similar to the results of Chen Haixia et al. [25], indicating that the up-regulated expression of structural genes promoted the synthesis of anthocyanin, but aluminum ions may not be the activators.

The expression of anthocyanin synthesis structural genes is also regulated by transcription factors [38]. At present, MYB, bHLH and WD40 are the main transcription factors regulating anthocyanin synthesis [41,42]. The expression levels of *CeMYB52* and *CeMYB104* in purple and red sepals were significantly higher than those in yellow and white sepals, and the expression levels were proportional to the anthocyanin content in sepals [43]. Under strong light, in the purple leaves of pepper (*Capsicum annuum* L.), *bHLH90-like* is significantly expressed and is highly positively correlated with anthocyanin synthesis genes [44]. The transcription factor *HymMYB2* may regulate the formation of the blue calyx by affecting the expression of *C3'5'H*, *DFR* and *ANS* in blue *Hydrangea* 'Endless Summer' [4]. This study found that two *MYB* (Hma1.2p1_0653F.1_g196210.gene, Hma1.2p1_0457F.1_g156980.gene) genes and *4CL* (Hma1.2p1_1457F.1_g284660.gene) were positively correlated; the *MYB* (Hma1.2p1_0547F.1_g176620.gene) and *bHLH* genes (Hma1.2p1_0628F.1_g192080.gene) were negatively correlated with *4CL* (Hma1.2p1_1457F.1_g284660.gene). It can be seen that the *MYB* gene and the *bHLH* gene may be involved in the expression of *4CL* gene as positive and negative regulators, respectively.

NAC transcription factors play an important role in plant growth and development, stress response and the regulation of secondary metabolite biosynthesis. A new NAC gene, *SmNAC1*, was cloned from *Salvia miltiorrhiza* [45]. The overexpression of *SmNAC1* enhanced the tolerance of transgenic plants to high zinc concentrations. It was also found that *PpNAC1* could promote anthocyanin accumulation in blood-fleshed peach by activating the expression of *PpMYB10.1* [46]; apple *MdNAC52* binds to *MdMYB9* and *MdMYB11* promoters to promote the accumulation of anthocyanins and proanthocyanidins [47]. The NAC (Hma1.2p1_1079F.1_g254920.gene, Hma1.2p1_1124F.1_g259533.gene) identified in this study was positively correlated with *4CL* (Hma1.2p1_1457F.1_g284660.gene) and *F3H* (Hma1.2p1_0956F.1_g241310.gene) and positively regulated *4CL* and *F3H* with two MYB genes. Therefore, the interaction between them deserves further study.

Plant glutathione transferase (GST) is a multifunctional family involved not only in the transport of anthocyanins [7,8,48] but also in primary and secondary metabolism in plant cells, resistance to heavy metal stress and other activities [49–51]. Glutathione transferase (GST), located in the cytoplasm, catalyzes the covalent binding of glutathione (GSH) and anthocyanin to form a glutathione cross-linked complex, which is recognized by MRP on the tonoplast and transported across the membrane to the vacuole [7,16]. GSTs related to anthocyanin transport have been isolated and identified in maize [8], petunia [7], Arabidopsis [52], perilla [53], grape [54], cyclamen [9] and carnation [55], cineraria [56] and Yunnan wild yellow peony [57]. In this study, it was found that the calyx color of the aluminum treatment group gradually became blue during the flower development, and the increase in anthocyanin was significantly higher than that of the control group. However, aluminum treatment did not up-regulate the expression of the middle and lower structural genes of anthocyanin synthesis. Therefore, it is believed that the difference in anthocyanin accumulation may be related to the enhancement of aluminum-induced anthocyanin transport capacity. Differential gene analysis showed that the anthocyanin transporter gene *AtGSTF12*, a homologous gene of *GST* (Hma1.2p1_0158F.1_g069560.gene), was up-regulated with the deepening of the calyx color, and the MRP protein genes *ABCC1* (Hma1.2p1_0021F.1_g014400.gene) and *ABCC2* (Hma1.2p1_0491F.1_g164450.gene) were also up-regulated. It has been identified that Arabidopsis *AtABCC1* and *AtABCC2* are metal chelate transporters located on the vacuolar membrane, which mediate the tolerance of cells to arsenic, cadmium and mercury [58]. *Vitis vinifera VvABCC1* can mediate the vacuolar membrane transport of anthocyanin [18]. *ZmMRP3* is necessary for the accumulation of anthocyanin in the aleurone layer of seeds [16]. Therefore, the function of *ABCC1* and *ABCC2* in *Hydrangea macrophylla* and the mechanism involved in anthocyanin transport deserve further study.

5. Conclusions

Exogenous aluminum treatment significantly increased the content of delphinidin-3-O-glucoside and Al^{3+} in the calyx of *Hydrangea macrophylla*, and 3 g/L aluminum treatment made the calyx blue. A total of 45,436 genes and 53 anthocyanin metabolites were identified by comparative analysis of the transcriptome and anthocyanin metabolome of the three stages of calyx color change. The contents of three main differential metabolites, delphinidin, delphinidin-3-O-glucoside and delphinidin-3-O-galactoside, increased in the 3 g/L aluminum treatment group and were positively correlated with the expression of *CHS* and *F3H* genes. However, only the expression of the upstream structural gene *4CL* was significantly up-regulated under aluminum treatment, and MYB and NAC may jointly positively regulate the expression of *4CL*. Aluminum induced a significantly up-regulated expression of the anthocyanin transporter gene *ABCC1* (Hma1.2p1_0021F.1_g014400.gene), *ABCC2* (Hma1.2p1_0491F.1_g164450.gene), as well as the aluminum transporter gene *ALS3* (Hma1.2p1_0111F.1_g053440.gene). This induction may be an important factor in promoting the transport of anthocyanin and aluminum ions to the vacuole, resulting in the blue color of the calyx. This study preliminarily clarified the effect of aluminum ions on

anthocyanin synthesis and metabolism, providing valuable ideas for further elucidating the formation mechanism of blue *Hydrangea macrophylla*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae10070745/s1>, Figure S1: Sample PCA and correlation heat map; Figure S2: Eight expression patterns of differentially expressed genes in group CK; Figure S3: Eight expression patterns of differentially expressed genes in the aluminum treatment group; Table S1: Metabolome sequencing-related parameter information; Table S2: Primers used for qRT-PCR in this study; Table S3: Changes in calyx floral color values under aluminum treatment; Table S4: All anthocyanins detected in the samples; Table S5: Expression of all differential genes; Table S6: All correlation data in this study.

Author Contributions: Conceptualization, H.C. and H.J.; methodology, W.L.; software, P.L.; validation, W.L., P.L. and H.Z.; formal analysis, T.Z.; investigation, W.L.; resources, H.C.; data curation, H.Z.; writing—original draft preparation, W.L.; writing—review and editing, H.C.; visualization, H.J.; supervision, T.Z.; project administration, H.C.; funding acquisition, H.C. All authors have read and agreed to the published version of the manuscript.

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

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