Identification of Seven Key Structural Genes in the Anthocyanin Biosynthesis Pathway in Sepals of Hydrangea macrophylla

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Abstract: Under specific cultivation conditions, the sepal color of Hydrangea macrophylla (H. macrophylla) changes from red to blue due to the complexation of aluminum ions (Al\(^{3+}\)), delphinidin 3-glucoside, and copigments. However, this phenomenon cannot occur in all cultivars despite the presence of sufficient Al\(^{3+}\) and copigments. To explore the mechanism of sepal bluing in H. macrophylla, there is an urgent need to study the molecular regulation of the anthocyanin biosynthesis pathway. However, the key structural genes, other than CHS, regulating anthocyanin biosynthesis in the sepals of H. macrophylla have not been identified. In this study, based on full-length transcriptome data from H. macrophylla ‘Bailmer’, the key structural genes regulating anthocyanin biosynthesis in the sepals of H. macrophylla were isolated and investigated. Ultimately, seven key structural genes, HmCHS1, HmCHI, HmF3H1, HmF3’H1, HmF3’5’H, HmDFR2, and HmANS3, were demonstrated to show high expression levels in colored sepals. The expression levels of these seven genes increased gradually with the development of sepals and were highest in the full-bloom stage. The trend of gene expression was consistent with the trend of anthocyanin contents. It was concluded that the seven selected genes were involved in anthocyanin biosynthesis in the sepals of H. macrophylla. The full-length sequence data have been deposited into the NCBI Sequence Read Archive (SRA) with accession number PRJNA849710. This study lays a good foundation for the further elucidation of the molecular mechanism of sepal coloration in H. macrophylla.

Keywords: full-length transcriptome; total anthocyanin Delphinidin 3-glucoside; gene expression

1. Introduction

Hydrangea macrophylla (H. macrophylla) is a deciduous shrub in the family Hydrangeaceae. H. macrophylla is a popular ornamental plant due to its large inflorescences, various flower colors, and long flowering period; therefore, it is widely used for cut flower production and as a potted or landscape plant. The sepals of H. macrophylla are its main ornamental organs [1]. The colors of H. macrophylla sepals vary from white to pink, red, purple, and blue, among which blue sepals are popular with consumers in the market. Interestingly, the development of blue sepals depends on cultivation conditions. In suitable acidic soil, aluminum ions (Al\(^{3+}\)) can be taken up by the roots and then transported into the vacuoles of sepals, where they form complexes with anthocyanins and copigments; thus, the sepal color changes from the original red coloration to purple and then blue [2–5]. In alkaline soil, aluminum exists as an Al(OH)\(_3\) precipitate and cannot be taken, so the sepals retain their original color of red or pink [6–8].

Because of this unique characteristic, the mechanism of sepal color formation has become a research hotspot. Initially, it was found that the anthocyanin 3-O-glucosyldelphinidin and the copigment 5-O-cafeoylquinic acid or 5-O-p-coumaroylquinic acid were involved in the development of blue sepals [9–13]. Then, it was reported that the vacuole pH value also played a key role in coloration, as the pH levels of the blue and red vacuoles were...
significantly different, at 4.1 and 3.3, respectively. When 3-O-glucosyldelphinidin, 5-O-
cafeoylquinic acid or 5-O-p-coumaroylquinic acid and Al3+ were mixed in buffer solution
at a ratio of 1:1:1 at pH 4.0, the blue color could be reproduced in vitro [14], revealing the
molecular structure of the blue metal complex pigment in H. macrophylla. A recent study
indicated that the blue cells in the sepals were mainly located in the second cell layer and
that the distributions of Al3+ and the H. macrophylla blue complex overlapped with the area
of blue cells, which confirmed that aluminum and the H. macrophylla blue-complex exist in
the blue cells of sepals and are involved in blue coloration [15].

Although the molecules included in the H. macrophylla blue complex have been identi-
fied, it is still difficult to regulate blue flower production, and not all cultivars can be
induced to develop a blue color under specific cultivation conditions. At present, the
molecular mechanism of flower color formation in sepals is still unclear.

In general, red and blue colors are determined by pelargonidins and delphinidins,
respectively [10,16]. However, in H. macrophylla, all sepal colors originate from a common
anthocyanin, 3-O-glucosyldelphinidin [9,14]. The anthocyanin biosynthesis pathway has
been well studied as an important pathway of a branch of the plant flavonoid pathway;
there are seven key structural genes in the anthocyanin biosynthesis pathway, including
the chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3-hydroxylase (F3H),
flavonoid 3-hydroxylase (F′3H), flavonoid 3-5 hydroxylase (F3′5′H), dihydroflavonol 4-
reductase (DFR), and anthocyanidin synthase (ANS) genes [17,18]. Although CHS, DFR,
and F3H have been reported to regulate anthocyanin biosynthesis in the sepals of H.
macrophylla, sequence information was found only for CHS [19–21]. To gain further insight
into the molecular mechanism of sepal coloration, it is essential to determine the key
structural genes regulating anthocyanin biosynthesis in the sepals of H. macrophylla. In
this study, based on full-length transcriptome data from H. macrophylla ‘Bailmer’, the
CHS, CHI, F3H, F′3H, F3′5′H, DFR, and ANS genes were selected as candidate genes, and the key
structural genes regulating anthocyanin biosynthesis in sepals were identified.

2. Materials and Methods

2.1. Plant Materials and Sampling

Three cultivars of H. macrophylla were used in this research, among which ‘Bailmer’
and ‘Duro’ are cultivars with pink sepals, whereas ‘Saxon Kleiner Winterberg’ has white
sepals. Under aluminum sulfate treatment, the sepal color of ‘Bailmer’ turned blue, while
that of ‘Duro’ and ‘Saxon Kleiner Winterberg’ remained pink and white, respectively.
These cultivars were planted in the greenhouse at the Institute of Vegetables and Flowers,
Chinese Academy of Agricultural Sciences (Beijing, China). Half of the ‘Bailmer’ plants
were treated with an aluminum sulfate solution (Al2(SO4)3·18H2O dissolved in water),
and the sepals turned blue (Tr group). The substrate mixture was soaked with the aluminum
sulfate solution 9 times, and each pot was soaked in approximately 20 g of aluminum
sulfate (Al2(SO4)3·18H2O) in total. The remaining half of the ‘Bailmer’ plants (CK group)
and all ‘Duro’ and ‘Saxon Kleiner Winterberg’ plants were grown without aluminum
sulfate treatment.

The roots, stems, leaves, terminal buds, lateral buds, pedicels, flowers, and sepals
were harvested at four developmental stages (S1: bud stage sepals, S2: discoloration stage
sepals, S3: full-bloom stage sepals, S4: senescence stage sepals) (Figure 1). All of these plant
materials were immediately frozen in liquid nitrogen and then stored at −80 °C.

2.2. Determination of Total Anthocyanin Contents

For ‘Saxon Kleiner Winterberg’, ‘Duro’, and ‘Bailmer’ (CK), the total anthocyanin con-
tents of the sepals in four developmental stages (S1, S2, S3, and S4) were determined using
a Plant Polyphenol-Chlorophyll Measuring Instrument (Dualex Scientific+, Orsay, France).
Figure 1. Materials of ‘Saxon Kleiner Winterberg’, ‘Duro’, and ‘Bailmer’. (a) Sepals in four developmental stages (S1, S2, S3, and S4) from ‘Saxon Kleiner Winterberg’; (b) roots, stem, leaf, and sepals in four developmental stages (S1, S2, S3, and S4) from ‘Duro’; (c) roots, stems, leaves, terminal buds, lateral buds, pedicels, and flowers from the CK and Tr groups of ‘Bailmer’ and sepals in four developmental stages (S1, S2, S3, and S4) from the CK group of ‘Bailmer’.

2.3. Full-Length Transcriptome Sequencing and Gene Annotation

Total RNA was extracted from the roots, stems, leaves, terminal buds, lateral buds, pedicels, and flowers of the CK group and Tr group of ‘Bailmer’ by grinding the tissue in TRIzol reagent (Life Technologies, Carlsbad, CA, USA) on dry ice and processing it according to the protocol provided by the manufacturer. An Agilent® 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) and a NanoDrop® series spectrophotometer (Thermo Scientific, Carlsbad, CA, USA) were used to determine the integrity and concentration of the RNA samples. The qualified RNA samples were mixed in equal amounts. mRNA was then enriched by using Oligo (dT) magnetic beads, and the enriched 5 μg mRNA was reverse transcribed into first-strand cDNA using the Clontech SMARTer PCR cDNA Synthesis Kit (Clontech, San Francisco, CA, USA). Then, PCR amplification was performed to synthesize double-stranded cDNA, and AMPure PB Beads (AMPure, Pasadena, CA, USA) were used to purify PCR amplification products. The SMRTbell template was annealed to a sequencing primer and bound to polymerase, and sequencing was conducted on the PacBio SequelII platform by Gene Denovo Biotechnology Co. (Guangzhou, China). The raw sequencing reads of the cDNA libraries were analyzed using an isoform sequencing (Iso-Seq) pipeline supported by Pacific Biosciences [22].

To annotate isoforms, they were subjected to BLAST searches against the NCBI non-redundant protein (Nr) database (http://www.ncbi.nlm.nih.gov, accessed on 2 December
2020), the Swiss-Prot protein database (http://www.expasy.ch/sprot, accessed on 4 December 2020), the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg, accessed on 7 December 2020), and the COG/KOG database (http://www.ncbi.nlm.nih.gov/COG, accessed on 9 December 2020). The BLASTx program (http://www.ncbi.nlm.nih.gov/BLAST/, accessed on 26 December 2020) was run with an E-value threshold of $1 \times 10^{-5}$ to evaluate sequence similarity with genes of other species.

2.4. Selection and Validation of Genes

2.4.1. Gene Selection

CHS, CHI, F3H, F3′H, F3′5′H, DFR, and ANS, which encode major enzymes in the anthocyanin biosynthesis pathway, were searched based on full-length transcriptome data. The open reading frames (ORFs) of the isoforms were analyzed online with NCBI ORF finder software (https://www.ncbi.nlm.nih.gov/orffinder accessed on 1 September 2022, NCBI, Bethesda, USA), and the multiple sequence alignment of isoforms was carried out using DNAMAN software.

2.4.2. qRT–PCR analysis

The roots, leaves, and sepals at S1, S2, and S3 in ‘Bailmer’, the roots, leaves, and sepals at S3 in ‘Duro’, and sepals at S3 in ‘Saxon Kleiner Winterberg’ from the CK group (without aluminum sulfate treatment) were harvested and were stored at $-80^\circ$C. All samples were ground to a powder in liquid nitrogen to extract total RNA using an RNA extraction kit (Huayueyang Biotechnology Inc., Beijing, China) according to the manufacturer’s instructions. The integrity and concentration of the RNA were analyzed by using 1% agarose gel electrophoresis and an Ultramicro ultraviolet-visible spectrophotometer (ND-100C, Miulab, Hangzhou, China), respectively. First-strand cDNA was synthesized using a PrimeScriptTM RT reagent kit with gDNA Eraser (Takara, Osaka, Japan) according to the manufacturer’s instructions.

The primer pairs targeting the genes were designed using Primer Premier 5.0 software (Premier, Ottawa, Canada) (Table 1). The EF1-β housekeeping gene (F- CGCAGCTGTTCGAGAAAGGC, R- GCGAGCTGCGAAGACACAGA) was used as the internal control for normalization. The cDNA template was fully mixed with Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) for qRT–PCR in a Light Cycler 480 System (Roche, USA). The qRT–PCR protocol was as follows: 95 $^\circ$C for 30 s, followed by 40 cycles of 95 $^\circ$C for 10 s, and 60 $^\circ$C for 30 s. Finally, melting curves were generated for all genes at 95 $^\circ$C for 15 s, 60 $^\circ$C for 60 s, and 95 $^\circ$C for 15 s. The relative expression levels of the target genes were calculated via the $2^{-\Delta\Delta C_q}$ method. One-way ANOVA (significant differences $p = 0.05$), Duncan’s method for multiple comparison tests, and Pearson correlation analysis were performed using SPSS 2.0 software (IBM, Armonk, USA).

Table 1. The structural genes in the anthocyanin biosynthesis pathway in *H. macrophylla* and the corresponding primer sequences.

| Protein Name | Gene Name | Family Member | Isoforms ID | Primer Sequence (5′-3′) | Amplicon Length |
|--------------|-----------|---------------|-------------|--------------------------|----------------|
| Chalcone synthase | CHS | HmCHS1 | Isoform0051878 | F-AATTTTCAGCGCATGTGTGACAATT R-CCACCACCATGTCTTGTCTAGC | 134 |
| | | HmCHS2 | Isoform0001712 Isoform0002955 | F-AGGGCGCACGTGTTCTTGTT R-GATCACCGCTGATGCACCGT | 125 |
| Chalcone isomerase | CHI | HmCHI | Isoform0062450 Isoform0064242 | F-ATTGTTCCTCGGTGGCGCAG R-GCCCTTCCACCTAACGCCAG | 124 |
### Table 1. Cont.

| Protein Name | Gene Name | Family Member | Isoforms ID | Primer Sequence (5'-3') | Amplicon Length |
|--------------|-----------|---------------|-------------|-------------------------|-----------------|
| Flavanone 3'-hydroxylase | F3H | HmF3H1 | Isoform002931 | F-AGGGATGGTGGAAACACTTGGR-TGCTCAAGATAATGCCATGGCTCG | 89 |
| | | HmF3H2 | Isoform0053472 | F-CTCTCTCTTACAGGGAGGTGGTG | 211 |
| | | HmF3H3 | Isoform0056122 | R-TCTCTGTTGACAGCATGGTGG | 135 |
| Flavonoid 3'-hydroxylase | F3'H | HmF3'H1 | Isoform0047984 | F-AGGCCCTTATTGAGTGGACAGTGGTG | 178 |
| | | HmF3'H2 | Isoform0013683 | R-TACGGGAGTTTTTGATGTGGGAC | 119 |
| | | | Isoform0048829 |isoform0042732 | isoform0047641 | F-GCAACTCCGATGCGTTTCCCTCTC | 151 |
| | | | Isoform0057874 | R-CAAGCACCAATCTCTTCTCCTCAAAGGAGGTGGTGGGC | 146 |
| | | | DFR | HmDFR1 | Isoform002980 | F-AGGCCATCCAGGGAGGTGGAC | 148 |
| | | | HmDFR2 | Isoform0054522 | R-TGCTCAAGATAATGCCATGGTGG | 134 |
| | | | Ans | HmANS1 | Isoform0064501 | F-AGGCCATCCAGGGAGGTGGAC | 104 |
| | | | HmANS2 | Isoform0002842 | R-CAAGCAAGCTTATCTCCTCCTCA | 98 |
| | | | | Isoform0004318 | F-TGTGATCAAAATACAGCTTCTAAGG | 134 |
| | | | HmANS3 | Isoform005422 | R-CTAGAGCGAGCTCCTGCTTG | 134 |

3. Results

3.1. Anthocyanin Contents

In the two cultivars with colored sepals (‘Bailmer’ and ‘Duro’), the contents of total anthocyanins in the sepals increased from the bud stage (S1) to the full-bloom stage (S3) and then decreased in the senescence stage (S4), with the highest accumulation of anthocyanins being observed at S3 (Figure 2). In ‘Saxon Kleiner Winterberg’ with white sepals, the anthocyanin content of the sepals remained low throughout their development.

![Figure 2](image-url) Contents of total anthocyanins in sepals at different developmental stages in ‘Saxon Kleiner Winterberg’, ‘Bailmer’ (CK), and ‘Duro’. Different lowercase letters indicating significant differences at the 0.05 level of probability according to Duncan’s multiple-range test.
3.2. Analysis of Full-Length Transcriptome Sequencing Data

A total of 72,848 high-quality isoforms were obtained from the full-length transcriptome sequencing data. A total of 67,941 (93.26%) of these isoforms were annotated in the Nr, SwissProt, KEGG, and KOG databases. The specific numbers of isoforms annotated in the NR, KEGG, KOG and SwissProt databases were 66,896 (91.83%), 65,418 (89.80%), 44,208 (60.69%), and 55,845 (76.66%), respectively (Figure 3).

3.3. Screening of Key Structural Genes Involved in Anthocyanin Biosynthesis

Thirty-seven isoforms related to structural genes in the anthocyanin biosynthesis pathway that showed similar sequences to CHS, CHI, F3H, F3′H, F3′5′H, DFR, and ANS were identified via NCBI BLAST searches. The sequences of isoforms showing more than 98% coverage and 98% identities in the CDS region were classified as belonging to the same gene. Thus, a total of 14 genes were obtained (Table 1). All of the structural genes except for CHI and F3′5′H had multiple family members. The specific primer pairs used for each gene are listed in Table 1.

3.4. Expression Pattern Analysis of Key Structural Genes Involved in Anthocyanin Biosynthesis

The H. macrophylla CHS gene family has two members, HmCHS1 and HmCHS2. The expression patterns of HmCHS1 in the roots, stems, leaves, and full-bloom stage sepals were different from those of HmCHS2. In both ‘Bailmer’ and ‘Duro’, the expression levels of HmCHS1 were the highest in the sepals and lowest in the roots and stems (Figure 4A), whereas the expression level of HmCHS2 in sepals was lower than that in the other organs (Figure 4B).

Subsequently, the expression levels of HmCHS1 in the pink sepals of ‘Bailmer’ and ‘Duro’ were significantly higher than those in the white sepals of ‘Saxon Kleiner Winterberg’. However, the expression levels of HmCHS2 in the sepals of ‘Bailmer’ and ‘Duro’ were lower than the level in ‘Saxon Kleiner Winterberg’ (Figure 4C).

The results indicated that HmCHS1 is a key structural gene involved in anthocyanin biosynthesis in sepals.

Only one CHI gene, named HmCHI, was obtained. In both ‘Bailmer’ and ‘Duro’, the expression levels of HmCHI were the highest in sepals among all the tested organs (Figure 5A). Moreover, the expression levels of HmCHI in the pink sepals of ‘Bailmer’ and ‘Duro’ were higher than those in the white sepals of ‘Saxon Kleiner Winterberg’ (Figure 5B). Therefore, HmCHI is the key structural gene involved in anthocyanin biosynthesis in sepals.
except for CHI and F3′5′H had multiple family members. The specific primer pairs used for each gene are listed in Table 1.

3.4. Expression Pattern Analysis of Key Structural Genes Involved in Anthocyanin Biosynthesis

The *H. macrophylla* CHS gene family has two members, *HmCHS1* and *HmCHS2*. The expression patterns of *HmCHS1* in the roots, stems, leaves, and full-bloom stage sepals were different from those of *HmCHS2*. In both ‘Bailmer’ and ‘Duro’, the expression levels of *HmCHS1* were the highest in the sepals and lowest in the roots and stems (Figure 4A), whereas the expression level of *HmCHS2* in sepals was lower than that in the other organs (Figure 4B).

Subsequently, the expression levels of *HmCHS1* in the pink sepals of ‘Bailmer’ and ‘Duro’ were significantly higher than those in the white sepals of ‘Saxon Kleiner Winterberg’. However, the expression levels of *HmCHS2* in the sepals of ‘Bailmer’ and ‘Duro’ were lower than the level in ‘Saxon Kleiner Winterberg’ (Figure 4C). The results indicated that *HmCHS1* is a key structural gene involved in anthocyanin biosynthesis in sepals.

![Figure 4](image4.png)

**Figure 4.** (A) Expression levels of *HmCHS1* in different organs; (B) expression levels of *HmCHS2* in different organs; (C) expression levels of *HmCHS1* and *HmCHS2* in sepals among the three cultivars. Different lowercase letters indicating significant differences at the 0.05 level of probability according to Duncan’s multiple-range test.

Only one CHI gene, named *HmCHI*, was obtained. In both ‘Bailmer’ and ‘Duro’, the expression levels of *HmCHI* were the highest in sepals among all the tested organs (Figure 5A). Moreover, the expression levels of *HmCHI* in the pink sepals of ‘Bailmer’ and ‘Duro’ were higher than those in the white sepals of ‘Saxon Kleiner Winterberg’ (Figure 5B). Therefore, *HmCHI* is the key structural gene involved in anthocyanin biosynthesis in sepals.

There are three members of the *H. macrophylla F3H* gene family, *HmF3H1*, *HmF3H2*, and *HmF3H3*. In both ‘Bailmer’ and ‘Duro’, *HmF3H1* presented the highest expression level in sepals (Figure 6A), while *HmF3H2* showed the highest expression level in leaves.

![Figure 5](image5.png)

**Figure 5.** (A) Expression levels of *HmCHI* in different organs; (B) expression levels of *HmCHI* in sepals among the three cultivars. Different lowercase letters indicating significant differences at the 0.05 level of probability according to Duncan’s multiple-range test.

![Figure 6](image6.png)
(Figure 6B). Although HmF3H3 exhibited the highest expression level in the sepals of ‘Bailmer’, it exhibited the highest expression level in the roots of ‘Duro’ (Figure 6C). In addition, the expression levels of HmF3H1 were significantly higher in pink sepals than in white sepals (Figure 6D). Therefore, HmF3H1 is a key structural gene involved in anthocyanin biosynthesis in sepals.

Figure 6. (A) Expression levels of HmF3H1 in different organs; (B) expression levels of HmF3H2 in different organs; (C) expression levels of HmF3H3 in different organs; (D) expression levels of HmF3H1, HmF3H2, and HmF3H3 in sepals among the three cultivars. Different lowercase letters indicating significant differences at the 0.05 level of probability according to Duncan’s multiple-range test.

Two members of the F3’H gene family were identified, HmF3’H1 and HmF3’H2. In ‘Bailmer’, the expression level of HmF3’H1 was lower in sepals than in roots and leaves, and in ‘Duro’, its expression was lower in sepals than in leaves (Figure 7A). HmF3’H2 presented the lowest expression level in sepals among all the tested organs (Figure 7B). Thus, the expression levels of HmF3’H1 and HmF3’H2 were not higher in sepals than in the other organs.

Furthermore, the expression levels of HmF3’H1 in the colored sepals of the two cultivars were significantly higher than those in the white sepals of ‘Saxon Kleiner Winterberg’. However, the expression level of HmF3’H2 in the pink sepals of ‘Duro’ was lower than that in the white sepals of ‘Saxon Kleiner Winterberg’ (Figure 7C).

In conclusion, HmF3’H1 is a key structural gene involved in anthocyanin biosynthesis in sepals.

Only one gene annotated as F3’5’H, named HmF3’5’H, was screened. The expression level of HmF3’5’H was the highest in sepals among all the tested organs (Figure 8A). The expression level of HmF3’5’H was significantly higher in colored sepals than in white
sepals (Figure 8B). Therefore, $HmF3'5'H$ is a key structural gene involved in anthocyanin biosynthesis in sepals.

**Figure 7.** (A) Expression levels of $HmF3'H_1$ in different organs; (B) expression levels of $HmF3'H_2$ in different organs; (C) expression levels of $HmF3'H_1$ and $HmF3'H_2$ in sepals among the three cultivars. Different lowercase letters indicating significant differences at the 0.05 level of probability according to Duncan’s multiple-range test.

**Figure 8.** (A) Expression levels of $HmF3'5'H$ in different organs; (B) expression levels of $HmF3'5'H$ in sepals among the three cultivars. Different lowercase letters indicating significant differences at the 0.05 level of probability according to Duncan’s multiple-range test.
Two DFR genes, HmDFR1 and HmDFR2, were found. The expression level of HmDFR1 was highest in sepals in ‘Bailmer’, whereas its expression was lower in sepals than in roots and stems in ‘Duro’ (Figure 9A). However, the expression levels of HmDFR2 were highest in sepals among all the examined organs of both ‘Bailmer’ and ‘Duro’ (Figure 9B).

Additionally, the expression level of HmDFR2 in colored sepals was higher than that in white sepals (Figure 9C). However, there was no significant difference in the expression level of HmDFR1 between colored sepals and white sepals.

It was concluded that HmDFR2 is a key structural gene involved in anthocyanin biosynthesis in sepals.

Similar to F3H, there were three identified members of the ANS gene family, HmANS1, HmANS2, and HmANS3. In ‘Bailmer’ and ‘Duro’, only HmANS3 showed a higher expression level in sepals than in the other organs (Figure 10A–C). Moreover, the expression level of HmANS3 was significantly higher in colored sepals than in white sepals (Figure 10D).

Therefore, HmANS3 is a key structural gene involved in anthocyanin biosynthesis in sepals.
Figure 10. (A) Expression levels of HmANS1 in different organs; (B) expression levels of HmANS2 in different organs; (C) expression levels of HmANS3 in different organs; (D) expression levels of HmANS1, HmANS2, and HmANS3 in sepals among the three cultivars. Different lowercase letters indicating significant differences at the 0.05 level of probability according to Duncan’s multiple-range test. In summary, according to the expression patterns observed in different organs, and the differences in expression levels between colored sepals and white sepals, seven genes, HmCHS1, HmCHI, HmF3H1, HmF3’H1, HmF3’5’H, HmDFR2, and HmANS3, were identified as key structural genes involved in anthocyanin biosynthesis in sepals.

### 3.5. Expression Patterns of the Seven Structural Genes Involved in Anthocyanin Biosynthesis during the Development of Sepals

In ‘Bailmer’ (CK) sepals, the expression levels of HmCHS1, HmCHI, HmF3H1, HmF3’H1, HmF3’5’H, HmDFR2, and HmANS3 increased gradually with the development of sepals (from S1 to S2, S3) and peaked at the full-bloom stage (S3) (Figure 11). The trend of gene expression was consistent with the trend of the anthocyanin contents (p < 0.01; Table 2). It was proven that the seven selected genes were related to anthocyanin biosynthesis in sepals.

### Table 2. Correlation coefficients between the expression levels of genes and the contents of anthocyanins.

|       | HmCHS1 | HmCHI | HmF3H1 | HmF3’H1 | HmF3’5’H | HmDFR2 | HmANS3 |
|-------|--------|-------|--------|---------|----------|--------|--------|
| Content of anthocyanins | 0.91<sup>1</sup> | 0.97<sup>1</sup> | 0.97<sup>1</sup> | 0.90<sup>1</sup> | 0.91<sup>1</sup> | 1.00<sup>1</sup> | 0.96<sup>1</sup> |

<sup>1</sup> Indicates a significant correlation at the 0.01 level.
Figure 11. Expression patterns of seven structural genes in the three developmental stages (S1, S2, S3) of sepals. Different lowercase letters indicating significant differences at the 0.05 level of probability according to Duncan’s multiple-range test.

4. Discussion

Anthocyanin accumulates in the vacuoles of petals and sepals and is the main pigment involved in flower color formation [7,23,24]. In the *H. macrophylla* ‘Meihong Mother’ cultivar, the anthocyanin content of sepals increases from the bud stage to the full-bloom stage and then decreases in the senescence stage. The accumulation of anthocyanin has also been reported to be highest in the full-flower stage in the ‘Thunb Ser’, ‘EndLess Summer’, and ‘Emile Mouillere’ cultivars [20,25]. In this study, the contents of the total anthocyanins in the colored sepals of ‘Bailmer’ and ‘Duro’ were similarly found to increase gradually from S1 to S2 and S3 and then decrease in S4 (Figure 2).

Although the genomics of *H. macrophylla* are being studied, and many complete or partial nucleotide sequences have been published in public databases [26], transcriptome sequencing remains an efficient method for mining genes. In our study, ‘Bailmer’ was selected for full-length transcriptome sequencing because of its unique characteristics, including blooming in new growth without a cold requirement, low-temperature resistance, and the development of blue flowers in acidic soil containing Al$^{3+}$. The full-length transcriptome database is helpful for exploring the mechanisms of flower color formation and aluminum detoxification in *H. macrophylla*. In the full-length transcriptome database, the CHS, F3H, F3′H, DFR, and ANS gene families showed multiple members, whereas CHI and F3′5′H did not (Table 1). Similar to many other higher plants, genome evolution has
produced gene families by gene duplication in this species [27]. In *Chrysanthemum*, there are multiple members of the structural gene families involved in the flavonoid biosynthesis pathway, with the exception of F3′5′H and ANS [28]. In *lily*, four unigenes have been annotated as CHS genes [29]. In the *H. macrophylla* ‘Forever Summer’ cultivar, multiple members have been identified for all key structural gene families except for CHI [30]. In this study, *HmCHS1, HmCHI, HmF3H1, HmF3′5′H, HmDFR2*, and *HmANS3* were selected as key structural genes participating in anthocyanin biosynthesis in sepals, and other members of their gene families may participate in flavonoid biosynthesis pathways in other tissues of *H. macrophylla*.

*CHS* [19], *DFR* [20], and *F3H* [21] have been reported to be structural genes related to anthocyanin biosynthesis in *H. macrophylla*. The sequence of *HmCHS1* identified in this experiment was highly similar (98.82%) to that of the *CHS* gene (AB011467.1) based on sequence comparison; thus, they should be identified as the same gene. However, there is a lack of sequence information about *F3H* and *DFR* in the literature, and we cannot determine whether the sequences of *HmF3H1* and *HmDFR2* selected in this study are the same as genes in previous reports.

Anthocyanins are the main compounds responsible for flower coloration, so the expression levels of structural genes regulating anthocyanin biosynthesis in sepals should be higher than those in other organs. All of the structural genes identified in this work except for *HmF3′H1* showed the highest expression levels in sepals. The two members of the F3′H family, both *HmF3′H1* and *HmF3′H2*, did not show higher expression in sepals than in other organs. A similar result was found in *Camellia Nitidissima Chi*, in which the expression level of the F3′H gene in petals was lower than that in leaves, whereas it was highest in fruits [31]. In *H. macrophylla*, *HmF3′H1* and *HmF3′H2* may play vital roles in other biosynthesis processes in leaves, stems, and roots.

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