Mechanism Underlying Color Variation in Calla Lily Spathes Based on Transcriptomic Analysis

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ABSTRACT. The calla lily (*Zantedeschia hybrida*) is a valued ornamental plant due to its unique shape and color variations. To determine the mechanisms responsible for color development in the calla lily spathe, we conducted a comparative transcriptomic analysis of the spathe of the black [Black Girl (B)], pink [Romantic (P)], and white [Ventura (W)] cultivars. The gene expression patterns in six spathe colors, including the preceding three colors as well as the amaranth [Promise (N)], red [Figo (F)], and yellow [Sun Club (Y)] cultivars were analyzed by real-time quantitative polymerase chain reaction (PCR). Transcriptomic analysis identified 25,165 differentially expressed genes. The transcription abundance and expression level of genes annotated as *anthocyanidin reductase* (*ANR1*, *ANR2*), *basic-helix-loop-helix* (*bHLH1*), and *glutathione S-transferases* (*GST1*) were significantly upregulated in B, and the expression of *anthocyanidin synthase* (*ANS*) was highest in B except for N. However, *chalcone isomerase* (*CHI2*) and *dihydroflavonol 4-reductase* (*DFR1*, *DFR2*) were expressed at significantly lower levels in P, W, and Y. Correlation analysis revealed that *bHLH1* might act as a positive regulator of *ANS* expression, promoting anthocyanin synthesis. Moreover, *GST1*-encoded proteins may be related to the accumulation and transport of both anthocyanin and procyanidin in the calla lily spathe. It is speculated that the formation of the black spathe is related to the accumulation of anthocyanins and procyanidins. However, the low expression of *CHI2*, *DFR1*, and *DFR2* may result in the inhibition of anthocyanin synthesis, which may lead to lightening of the spathe color. This preliminary study revealed the mechanism responsible for calla lily spathe color, identifying the key genes involved, thus providing effective gene resources and a theoretical basis for flower color molecular breeding.

The flower color of ornamental plants has attracted much attention (Qu et al., 2018). Research on the mechanisms of color formation in nonmodel ornamental plants can lead to a better understanding of plant color-related genetic resources (Tripathi et al., 2018). Flavonoids, carotenoids, and alkaloids provide different coloration to flowers (Hanbali et al., 2013; Kelebek and Selli, 2011). Among these, anthocyanins are one of the most important chromogenic substances in the metabolism of flavonoid compounds in higher plants (Bueno et al., 2012; Tripathi et al., 2018). Their biosynthesis is catalyzed by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), dihydroflavonol 4-reductase (DFR), ANS, anthocyanidin reductase (ANR), and other enzymes (Grotewold, 2006; Koes et al., 2005; Nguyen et al., 2006; Nováková et al., 2006; Sheng et al., 2018; Tanaka and Ohmiya, 2008). For example, the color variation in *Brunfelsia acuminata*, *Oncidium*, and *Magnolia sprengeri* is a consequence of differences in the expression of genes within the anthocyanin biosynthesis pathway, leading to variations in anthocyanin accumulation (Li et al., 2016; Liu et al., 2012; Shi et al., 2014). In addition, it has been reported that the pigmentation of brown fibers may be due to oxidized-state procyanidins (condensed tannins) and procyanidin (PA) precursors, and high *ANR* expression leads to greater PA accumulation (Kitamura et al., 2004). At the same time, the development of flower color

also depends on the influence of copigmentation, metal ions, and vacuole pH (Ohmiya, 2010). Differences in the expression of structural genes can lead to variations in anthocyanin accumulation, highlighting the likely roles played by transcription factors in plant color variations. In general, anthocyanin synthesis in plants is regulated by the Myb, bHLH, and WD40 transcription factors, as well as some protein complexes (such as MBW, composed of three transcription factors) that bind to the promoter regions of structural genes (Koes et al., 2005; Lepiniec et al., 2006; Ramsay and Glover, 2005; Saito et al., 2013; Xu et al., 2015). It has been found that anthocyanin transport and accumulation also affect flower color in plants. Anthocyanins are synthesized in the endoplasmic reticulum and are transported to the vacuole by glutathione S-transferases (GST), members of the multidrug and toxic compound extraction (MATE) family, and ATP-binding cassette (ABC) transporters (Francisco et al., 2013; Luo et al., 2018). The transport of PAs by GSTs has also been previously confirmed. For example, it has been found in *Arabidopsis thaliana*, *VviGST3*, and *VviGST4* may play a role in PA transport in the testa (Ricardo et al., 2016).

High-throughput transcriptome sequencing technology has revealed molecular mechanisms in specific biological processes that enable the study of overall gene function and gene structure (Zheng et al., 2015). At present, transcriptome sequencing has been successfully applied to studying the color patterns in many nonmodel plants, greatly improving our understanding of chromogenic genes and their expression patterns (Lin et al., 2018). For example, transcriptomics and differentially expressed gene analysis of the white and purple butterfly orchid (*Phalaenopsis amabilis*) and the white and pink tea flower (*Camellia sinensis*) were conducted to obtain candidate genes (structural and regulatory) involved in anthocyanin biosynthesis, revealing the

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mechanisms underlying petal color (Meng et al., 2019; Zhou et al., 2020). Therefore, differences in the abundance of color-related genes in anthocyanin biosynthesis pathways of different species may lead to flower color variation.

The calla lily is native to the family Araceae (X. Lei et al., 2017; Singh et al., 1996). It is one of the most popular ornamental plants due to its unique shape and varied colors. Previously, we evaluated the pigment components in the spathe of 27 calla lily cultivars imported from the Netherlands and found that the primary color determinants in the spathe were flavonoids that differed significantly in terms of both total anthocyanin and total flavanol content among the different colors (X. Lei et al., 2017). Previous studies on the calla lily mainly focused on tissue culture, analysis of main chromogenic substances in spathe (X. Lei et al., 2017; Lewis et al., 2003), and genetic diversity (Wei et al., 2016). However, little research has been done on the mechanism of color formation of the spathe.

In this study, transcriptome analysis was carried out on three different-colored calla lily cultivars: black [Black Girl (B)], pink [Romantic (P)], and white [Ventura (W)]. The underlying color mechanism was discussed in combination with the previous studies on the changes in the total anthocyanin content (TAC) (X. Lei et al., 2017). At the same time, qRT-PCR expression analysis was performed on the preceding three cultivars, and three additional colors, including amaranth [Promise (N)], red [Figo (F)], and yellow [Sun Club (Y)]. Finally, the differentially expressed genes (DEGs) that determined spathe color development in the calla lily were screened. This study provides a theoretical basis and reference for the identification, cloning, and functional analysis of the key genes underlying the development of color in calla lily spathes.

Materials and Methods

PLANT MATERIALS. Comparative transcriptomic analysis was conducted on six calla lily cultivars (Fig. 1), namely, B, P, W, Y, N, and F. Transcriptomic sequencing was carried out for the spathes of B, P, and W, and the qRT-PCR expression patterns of

Fig. 1. Six calla lily cultivars with different spathe colors: Black Girl (B), Romance (P), Ventura (W), Sun Club (Y), Promise (N), and Figo (F).

anthocyanin synthesis-related genes were analyzed for all cultivars. When the spathes were fully open, we collected the test materials at 1500 hr. Three individual plants were sampled from each cultivar and 0.2 g of the uniformly colored part of the spathe was removed for flash-freezing in liquid nitrogen. Samples were stored at -80°C until further use.

RNA EXTRACTION, COMPLEMENTARY DNA LIBRARY CONSTRUCTION, AND TRANSCRIPTOME SEQUENCING. Total RNA was extracted from spathe using an RNA extraction kit (0416-500; Beijing Huayueyang Biological Technology Co., Beijing, China) according to the manufacturer's protocol. The RNA quality was assessed on a bioanalyzer (model 2100; Agilent Technologies, Palo Alto, CA) and confirmed using RNase-free agarose gel electrophoresis (Singh et al., 2015). Following the total RNA extraction, the eukaryotic messenger RNA (mRNA) was enriched using Oligo (dT) (E7530; New England Biolabs, Ipswich, MA) beads, and prokaryotic mRNA was enriched by removing ribosomal RNA using the a Ribo-ZeroTM Magnetic Kit (Epicenter, Madison, WI). The enriched mRNA was then fragmented using fragmentation buffer and reverse-transcribed into complementary DNA (cDNA) with random primers. Second-strand cDNA was synthesized using DNA polymerase I, RNase H, dNTP, and buffer. The cDNA fragments were then purified using a PCR extraction kit (QiaQuick; Qiagen, Venlo, The Netherlands), end-repaired, poly(A) added, and ligated to Illumina (San Diego, CA) sequencing adapters (Fu et al., 2014). The target bands were then size-selected for PCR (Hu et al., 2015), and sequenced using the Illumina HiSeqTM2000 platform by Gene Denovo Biotechnology Co. (Guangzhou, China). The reads per kilobase per million mapped reads (RPKM) was used to represent the expression levels of different genes.

DE NOVO ASSEMBLY OF TRANSCRIPTOME SEQUENCING DATA AND FUNCTIONAL ANNOTATION. "Single genes" are genes identified by de novo assembly results and the sequence assembly quality is assessed using sequence and base number, length distribution, average coverage, and N50 statistics (Cock et al., 2009). Reads obtained from the sequencing machines include raw reads containing adapters or low-quality bases that will affect the following assembly and analysis. Thus, to get high-quality clean reads, reads were further filtered by fastp [version 0.18.0 (Chen et al., 2018)]. The data were cleaned by removing reads with the following parameters: 1) reads containing adapters; 2) reads containing more than 10% of unknown nucleotides (N); 3) low-quality reads containing more than 50% of low-quality bases [probability that the hypothesis is correct, but rejected = the number of negatives/the total number, which is a test probability against the sample data ($Q\text{-value} \leq 20$)]. The high-quality Illumina reads from three different cDNA libraries of the same cultivar were pooled to produce a calla lily transcriptome database, which was assembled de novo using Trinity software (Grabherr et al., 2011). Transcriptome databases of B, P, and W were constructed.

DEG ANALYSIS AND FUNCTIONAL ENRICHMENT. Single-gene expression was calculated and normalized to RPKM. RNA differential expression analysis was performed by DESeq2 (Love et al., 2014; Robinson et al., 2009) software between two different groups. The genes/transcripts with the parameter of false discovery rate ($\text{FDR} < 0.05$ and absolute fold change ($\text{FC} \geq 2$) were considered as differentially expressed genes/transcripts. The basic annotation of unigenes included protein functional annotation, pathway annotation, clusters of orthologous groups (COG), eukaryotic orthologous groups (KOG) functional annotation, and

gene ontology (GO) annotation. To annotate the unigenes, we used the BLASTx (Altschul et al., 1990) program with an E-value $< 1.0 \times 10^5$, the National Center for Biotechnology Information (NCBI) (Nuala et al., 2015) nonredundant protein (Nr) database (Guenter et al., 1998), the SwissProt protein database (Consortium, 2020), the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Ogata et al., 1999), and the COG/KOG database. Protein functional annotations were then obtained according to the best alignment results (Tatusov et al., 2003). The calculated probability was FDR-adjusted, taking $FDR \leq 0.05$ as a threshold. GO and KEGG terms meeting this condition were defined as significantly enriched in the DEGs. This analysis identified the primary biological functions of the DEGs. This method was further used to explore the mechanism of color change among different cultivars, different plant tissues, plants at different stages of development, or different treatments (Chen et al., 2012a; Jin et al., 2016; Li et al., 2010). Our study investigated the color variation mechanisms of calla lily spathes according to the enrichment pathways and the differential genes in the B, P, and W pathways and the significant differences in anthocyanin and flavonol content previously reported by our research group (X. Lei et al., 2017).

qRT-PCR-BASED VERIFICATION AND ANALYSIS. For exploring the highly F gene expression patterns in different cultivars, the expression of DEGs related to flavonoid biosynthetic and transcriptional pathways was tested using qRT-PCR. Nineteen unigenes involved in the anthocyanin metabolism pathway were selected for qRT-PCR analysis to verify the expression results determined by RNA-sequencing. All analyses were done in triplicate on three biological replicates, and the specific primers are listed in Supplemental Table 1. qRT-PCR was performed using SYBR Premix Ex Taq Perfect Real Time (TaKaRa, Tokyo, Japan) and using a sequence detector (Applied Biosystems ABI Prism 7500; Thermo Fisher Scientific, Waltham, MA). The expression patterns of key genes in the anthocyanin metabolism pathway in the spathe were verified. qRT-PCR was performed using the following conditions: pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at the optimum temperature for 30 s, extension at 60 °C for 30 s, for 40 cycles. The relative gene expression levels were calculated using the delta-delta Ct method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001), with *Cm-Actin* and *β -Tubulin* expression used as internal standards (repeated in triplicate). Finally, to better analyze the relationship between key genes and total anthocyanin, SPSS software (IBM SPSS Statistics version 24.0; IBM Corp, Armonk, NY) was used to calculate the correlation between the expression levels of key genes and total anthocyanin content, which is the Pearson correlation coefficient (R^2). The closer R^2 is to 1, the stronger is the correlation.

Results

SEQUENCING AND DE NOVO ASSEMBLY OF TRANSCRIPTOME DATA. To further understand the underlying molecular mechanisms of spathe color in the calla lily, three libraries representing B, P, and W were constructed. After trimming the adapters and filtering out low-quality reads ($N\% > 10\%$), 73,510,006 reads from B, 41,661,782 reads from P, and 37,623,484 reads from W were collected. The GC content was 50.5%, 50.55%, and 51.05% for B, P, and W, respectively, and the Q20 ratio (test error $< 1\%$) was 96.60%, 96.86%, and 97.83%, respectively. The sequencing results showed that the GC content was $\approx 50.5\%$ (Table 1), and the unknown base sequence fragment N of the three cultivars

Table 1. Transcriptome sequencing original read and assembly data for calla lily cultivars.

Cultivar	No.	Q20 (%) ^z	GC (%) ^y
Black Girl	73,510,006	96.6	50.51
Romantic	41,661,782	96.86	50.55
Ventura	37,623,484	97.83	51.05

^zProportion of guanine and cytosine to all bases in nucleic acid.

^yQuality control value of sequencing.

analyzed by transcriptome was 0 in all cases, indicating that the sequencing quality was high and further analysis could be carried out. The high-quality reads in the transcriptome database contained a total of 85,907 sequences, with an average transcript length of 785 base pairs (bp), among which the longest sequence was 15,804 bp and the shortest was 201 bp (Table 2, Fig. 2). Among these, 95.5% of the sequences were within 200 to 3000 bp (Fig. 2).

FUNCTIONAL ANNOTATION AND CLASSIFICATION. For an overall understanding of all unigene sequence-function information, the assembled transcriptome data were compared and annotated in the Nr, SwissProt, KEGG, KOG, and GO databases. Approximately 85,907 assembled unigenes were functionally annotated using BLASTx against the NCBI Nr, SwissProt, Protein family (Pfam), KOG, GO, and KEGG databases. From this, 29,816 (34.7%) transcripts were annotated to Nr, 20,897 (24.3%) to SwissProt, 10,322 (12%) to KEGG, and 18,034 (20.1%) to KOG (Fig. 3). Because of the lack of a complete calla lily genome sequence, the unigenes showed top matches to *Elaeis guineensis* at 24.4% similarity, followed by *Phoenix dactylifera* (20.5%), and *Nelumbo nucifera* (12.8%) (Fig. 4).

GO FUNCTIONAL ANNOTATION AND KEGG PATHWAY ENRICHMENT OF DEGs. To identify candidate genes related to anthocyanin biosynthesis, B, P, and W DEGs in calla lily spathes were analyzed by pairwise comparisons (P vs. B, W vs. B, W vs. P). A total of 25,165 DEGs were identified in the calla lily library, including 10,130 upregulated genes and 15,035 downregulated genes. There were 8880 significantly DEGs in P vs. B, including 3465 upregulated and 5235 downregulated genes. In W vs. P, 8395 genes were significantly differentially expressed, including 6162 that were upregulated and 2233 that were downregulated. In W vs. B, 7890 genes were significantly differentially expressed, including 5228 upregulated and 2662 downregulated genes (Fig. 5).

We annotated the GO function of DEGs obtained by comparison (Fig. 6). GO functional annotation indicated that DEGs were divided into 45 functional groups within biological processes, cellular components, and molecular functions. In the biological process category, most DEGs were classified as metabolic processes (10,440) and cellular processes (9759), followed by organic metabolic processes (8086). In the cell component category, cell (7462) and cell part (7461) were relatively dominant, followed by organelles (7147) and membrane group (7083). Among the molecular functions, catalytic activity (9448) and binding activity (9252) were the most representative.

Table 2. Assembly of sequencing results in calla lily.

Genes (no.)	Max length (bp)	Max length (bp)	Avg length (bp)	N50 (bp) ^z
85,907	15,804	201	785	1,557

^zQuality of genome splicing. bp = base pair.

Fig. 2. Assembled unigene length distribution of calla lily.

Although the comparison of the three cultivars showed similar GO functional classification patterns, they differed in enrichment classification (Supplemental Table 2). GO enrichment analysis showed that genes associated with the design and color of phenylalanine and flavonoid biosynthesis were significantly enriched. In P vs. B, 12, 11, and 2 unigenes were annotated to the phenylalanine biosynthesis pathway, flavonoid biosynthesis pathway, and flavonoid flavonol biosynthetic pathway, respectively. In W vs. B and W vs. P, there were 12 and 10 significantly enriched unigenes annotated as involved in phenylalanine biosynthesis pathways, 8 and 13 unigenes in flavonoid biosynthesis pathways, and 1 unigene potentially involved in the flavonol biosynthesis pathway, respectively.

The identification of DEGs in the anthocyanin biosynthesis pathway related to different-colored spathe can provide important information for the mechanism of color development in the calla lily. The primary methods of color development in the spathe of the three cultivars were further investigated by KEGG pathway analysis. It was found that the DEGs were divided into the following 10 categories and 34 KEGG terms: amino acid metabolism, biosynthesis of other secondary metabolites, carbohydrate metabolism, energy metabolism, environmental adaptation, global and overview, lipid metabolism, metabolism of cofactors and vitamins, metabolism of other amino acids, metabolism of terpenoids and polyketides, and signal transduction (Fig. 7). Comparing the

Fig. 3. Venn diagram of unigene annotation using nucleotide sequence database (Nr), clusters of orthologous groups for eukaryotic complete genomes (KOG), Kyoto Encyclopedia of Genes and Genomes (KEGG), and SwissProt protein sequence database (SwissProt) databases.

Fig. 4. Species taxonomy based on the nucleotide sequence database (Nr) database.

three cultivars, most DEGs were found in the carbohydrate metabolism category, followed by global and overview. We were particularly interested in this pathway and in identifying the specific genes involved, because the color of the spathe is influenced by the expression of numerous genes involved in the anthocyanin biosynthesis pathway, leading to differences in the type and content of anthocyanin. Therefore, enrichment analysis of DEGs mapped to KEGG biopathways was carried out to clarify the enrichment in biological function of each comparison group of DEGs (Supplemental Table 3). The identified DEGs were mapped to 12 genes in the “biosynthesis of other secondary metabolites” pathway, four of which are related to the flower color formation process. The flavonoid biosynthesis pathway contained the most significant DEG enrichment, followed by the phenylalanine biosynthesis pathway. Specifically, there were 843 DEGs annotated to 119 KEGG metabolic pathways in P vs. B. Among them, 35 DEGs were annotated to the phenylalanine biosynthesis pathway (with 28 upregulated and seven downregulated), and 13 DEGs were annotated to the flavonoid biosynthesis pathway (nine upregulated and four downregulated), which were significantly enriched. Three DEGs were related to flavonol biosynthesis (all downregulated), and one DEG was related to anthocyanin

Fig. 5. Analysis of global gene expression among three calla lily cultivars: Black Girl (B), Romance (P), and Ventura (W).

Fig. 6. Gene ontology (GO) classification of calla lily unigenes.

biosynthesis (upregulated). There were 842 DEGs annotated to 123 KEGG metabolic pathways in W vs. B. Among them, 31 were annotated to the phenylalanine pathway (23 upregulated and eight downregulated), 13 were annotated to the flavonoid biosynthesis pathway (11 upregulated and two downregulated), and three DEGs annotated to anthocyanin biosynthesis pathway (all downregulated), all of which were significantly enriched. In addition, one DEG was significantly related to flavonol biosynthesis. Among them, 32 (12 upregulated and 20 downregulated) DEGs were annotated to phenylalanine biosynthesis pathway, 11 (seven upregulated and four downregulated) DEGs were annotated to flavonoid biosynthesis pathway, four DEGs (all upregulated) were annotated to flavonoid biosynthesis pathway, and three DEGs (one upregulated and two downregulated) annotated. By means of DEG identification of anthocyanin biosynthesis pathway, the key genes of different colors of spathe were screened out and their coloring mechanism was analyzed. These DEGs laid a foundation for further research on the metabolism of flavonoids and related functional genes in the spathe of calla lily.

Fig. 7. Kyoto Encyclopedia of Genes and Genomes (KEGG) function classification of calla lily unigenes.

IDENTIFICATION OF DEGS AND KEY GENES RELATED TO THE ANTHOCYANIN BIOSYNTHESIS PATHWAY. To analyze the genes related to chromogenic substances in anthocyanin biosynthesis in the calla lily spathe [based on the RPKM values of *Clematis florida* (Kulahoglu et al., 2014)], 65 DEGs were screened to generate heatmaps (RPKM >30) and their log2 (RPKM) values were classified into three groups (Fig. 8, Supplemental Table 4). Cluster I was composed of 19 DEGs, namely *CHS*, *CHI1*, *CHI2*, *DFR3*, *A3GT1*, seven *Myb* (*Myb2*, 5, 8, 9, 11, 13, 14), four *bHLH* (*bHLH2*, 3, 12, 13), and three *WD40* (*WD40* 7, 9, 10). The expression of these genes was downregulated in 'P,' and the structural gene *CHI2* was the most downregulated (Fig. 8B Cluster I). There were 36 genes in Cluster II including *F3H*, *F3'H1*, *F3'H2*, *DFR1*, *DFR2*, *ANS*, *ANR1*, *ANR2*, *A3GT2*, nine *Myb* (*Myb1*, 3, 4, 6, 7, 10, 12, 15, 16), six *bHLH* (*bHLH1*, 5, 6, 8–10), six *WD40* (*WD40* 1–4, *WD40* 6, 8), and four *GST* (*GST1*, 3, 7, 8). The expression of structural genes in Cluster II was upregulated in B except that *DFR2* was significantly downregulated (Fig. 8B Cluster II). The Cluster III genes included two modifier genes (*A3*, *5GT1*, 2), four transcription factors (*bHLH4*, 7, 11; *WD40* 5), and four transporters (*GST2*, 4–6) that were downregulated in 'B.'

Based on the functional annotation and hierarchical clustering of the log2 (RPKM) value, combined with the related map and DEG enrichment pathways identified by GO and KEGG, we screened the genes that may play a key role in the color of calla lily spathe. All these DEGs contained both upstream and downstream genes (Fig. 9). For example, compared with B, *CHI2* transcriptional abundance was significantly downregulated (by 131%) in P, whereas *DFR1* and *DFR2* were significantly downregulated in W (by 126.5% and 560.8%, respectively). The transcriptional abundance of *ANS*, *ANR1*, and *ANR2* in B were significantly higher than in W by 312.3%, 327.9%, and 624.6%, respectively, and higher than in P by 52.1%, 347.6%, and 728.8%, respectively. The RPKM values of these six structural genes were significantly different among different cultivars. In

Fig. 8. (A) Expression profiles of anthocyanin biosynthetic genes differentially expressed in Romance (P), Black Girl (B), and Ventura (W) of calla lily cultivars. (B) Accumulation patterns of genes in three clusters. Data represent the log2 reads per kilobase per million (RPKM) mapped reads. The mean for each cluster is shown as a red line, and the expression patterns of individual genes are shown as gray lines.

addition, the transcription factor abundance of *bHLH1* in B was higher than in W and P by 856.6% and 371.3%, respectively. The abundance of the transporter *GST1* in B was higher than in W and P by 533.6% and 951.1%, respectively.

VERIFICATION AND EXPRESSION ANALYSIS OF CANDIDATE GENES. The anthocyanin composition and content in the six different-colored spathes were previously determined and analyzed. It was found that W and Y did not contain anthocyanins, P contained very few anthocyanins, and B, N, and F contained a large number of anthocyanins and flavonols, with B containing the most total anthocyanins, followed by N (X. Lei et al., 2017).

To verify the reproducibility and accuracy of the expression of DEGs identified by transcriptome analysis, we used qRT-PCR to determine the expression pattern of P, B, and W. Using qRT-PCR, we identified 11 structural genes *CHS*, *CHI2*, *F3H*, *F3'H1*, *F3'H2*, *ANS*, *ANR1*, *ANR2*, *DFR 1*, *DFR 2*, and *FLS*; one transporter *GST1*; and six transcription factors *Myb2*, *Myb5*, *bHLH1*, *bHLH5*, *bHLH6*, and *WD40 1*. The expression patterns of *CHS*, *CHI2*, *F3'H1*, *F3'H2*, *DFR1*, *DFR2*, *ANS*, *ANR1*, *ANR2*, *A3GT2*, *Myb2*, *bHLH1*, *bHLH6*, *WD40 1*, and *GST1* were highly consistent with the results of the high-throughput sequencing (Fig. 10).

To further analyze the color mechanism of the calla lily spathe, the expression patterns of 19 genes in the six colored spathes (P, B, W, Y, N, and F) were analyzed showing that the expression of these 19 genes differed significantly among the different colors. These genes were highly expressed in B, N, and

F with high anthocyanin content but at very low levels in W, P, and Y that contained little or no anthocyanin. Analysis of the gene expression levels in B, N, and F showed that *ANR1*, *ANR2*, *bHLH1*, and *GST1* were the most strongly expressed in B, whereas the expression levels of the other structural genes *ANS*, *CHS*, *F3H*, *F3'Hs*, and *DFRs* were lower than in N. We found that the transcription factor *bHLH1* and transporter *GST1* had RPKM values >100 in B and with a 5-fold difference in RPKM in W (which belongs to Cluster II), suggesting *ANS* may be regulated by *bHLH1* to promote anthocyanin synthesis (Fig. 8). Correlation analysis showed that the *bHLH1* expression level was associated with increases in both the total anthocyanin content and *ANS* ($R^2 = 0.995$ and 0.892 , respectively). Moreover, *GST1* showed strong correlations with *ANR1* and *ANR2* ($R^2 = 0.997$ and 0.997 , respectively). Interestingly, we also found that *GST1* was highly correlated with the total anthocyanin content ($R^2 = 0.994$). In addition, further analysis of the expression patterns of W, P, and Y showed the lowest expression level in the light colors, with trace expression of *DFR 1* and *DFR 2* in W but the highest expression of *CHI2* in F.

Discussion

OVERVIEW. The biological regulation of flower color has attracted much attention in the study of ornamental plants (Lin et al., 2019). Anthocyanin is known to contribute to plant

Fig. 9. Putative genes in the anthocyanin synthesis pathway and their expression levels. Heatmaps were constructed based on reads per kilobase per million mapped reads (RPKM) of pink, black, and white calla lily cultivars.

coloration (Rusishvili et al., 2019). In our study, we used spathes of the ornamental calla lily to identify the key genes underlying the color differences in the spathes and further analyzed the color mechanism. Taken together, our sequencing data provided an effective basis for the research of flower color gene resources for nonmodel plants.

FORMATION OF BLACK CALLA LILY SPATHES. Black flowers, fruits, leaves, seeds, and seedcoats are naturally found in various plant species (Markham et al., 2004). Previous studies on flower color development in the dahlia (*Dahlia pinnata*) suggest that a lack of flavonoids leads to a high accumulation of anthocyanins. Inhibition of *DvFNS* expression eliminates the competition between flavonoid and anthocyanin synthesis, eventually leading to the accumulation of anthocyanins (Davies et al., 2003; Deguchi et al., 2015). In our previous study, we found a high accumulation of anthocyanins and flavonols in the spathes of B, and section microstructure showed that the pigment was widely distributed in the two to three layers of mesophyll cells in the upper and lower epidermis of B (X. Lei et al., 2017), leading to the formation of the black spathe.

ANS is a key enzyme in anthocyanin synthesis, which catalyzes the conversion of colorless anthocyanin into colored anthocyanin (Shi et al., 2018). For example, in sprenger's magnolia (*Magnolia sprengeri*) it was found that a higher transcription level of *MsANS* led to anthocyanin accumulation (Shi et al., 2014). It has been previously reported that the high *ANS* expression in black rice (*Oryza sativa* 'Hanzhong') results in the accumulation of anthocyanin, giving a purplish-black grain appearance of the caryopsis (Yin et al., 2017). According to the qRT-PCR results, the expression of *ANS* in B was higher than in N, possibly due to the dependency of the red spathe formation on anthocyanin accumulation, whereas the formation of black spathes may not only be caused by the accumulation of a large amount of anthocyanin.

Therefore, we speculated that there may be other mechanisms involved in the formation of the black spathe. Interestingly, the expression levels of *ANR1* and *ANR2* in the black spathe were the highest among the six cultivars. Previous studies have shown that *ANR* is a key gene that catalyzes the transformation of anthocyanin into epicatechin (Li et al., 2019). Therefore, we

Fig. 10. Expression analysis of 19 genes related to anthocyanin biosynthesis in calla lilies using real-time quantitative polymerase chain reaction, including 12 structural genes (*CHS*, *CHI2*, *F3H*, *F3'H1*, *F3'H2*, *DFR1*, *DFR2*, *ANS*, *ANR1*, *ANR2*, *FLS*, and *A3GT2*), one transporter (*GST1*), and six transcription factors (*Myb2*, *Myb5*, *bHLH1*, *bHLH5*, *bHLH6*, and *WD40 1*). The Y-axis on the left represents the relative expression and on the right represents gene abundance; the X-axis depicts the six cultivars of calla lily, which are Black Girl (B), Romance (P), Ventura (W), Sun Club (Y), Promise (N), and Figo (F).

speculated that the high expression of *ANR1* and *ANR2* may lead to the accumulation of multiple procyanidins with possible interaction between anthocyanins and PAs, promoting the color deepening of the spathe. For example, experiments on the leaves of B, tobacco (*Nicotiana tabacum*), and Arabidopsis showed that there was an interaction between anthocyanin and PAs, and the interaction with PAs appeared to produce deeper color when procyanidin accumulation was high (Kitamura et al., 2004; Xie 2003). Large amounts of procyanidins and anthocyanins have been previously detected in the caryopses of black rice granules (Finocchiaro et al., 2010). These results support our hypothesis about the formation of the black spathe. In addition, the brown fibers of mature Arabidopsis and cotton (*Gossypium hirsutum*) may contain oxidized PAs (condensed tannins) and PA precursors (Debeaujon et al., 2001; Feng et al., 2013; Kitamura et al., 2004; Li et al., 2013; Tan et al., 2013; Xiao et al., 2007, 2014). Therefore, we believe that PA oxidation may also further promote the formation of the black spathe and we intend to investigate this in the future.

THE INHIBITORY EFFECTS OF CHI AND DFR PLAY IMPORTANT ROLES IN THE FORMATION OF LIGHT-COLORED SPATHES IN THE CALLA LILY. This study suggested that the low expression of *CHI2*, *DFR1*, and *DFR2* plays a key role in the formation of the pink or white spathe. Chalcone isomerase (CHI) is a stable enzyme involved in the early biosynthesis of anthocyanin, and promotes the molecular internalization of chalcone to form flavonoids (Nishihara et al., 2005; Wu et al., 2018). DFR catalyzes dihydroxyflavone alcohols (DHK, DHQ, and DHM) to colorless anthocyanins (LPG, LCY, and LDP) and has selective specificity for the three dihydroxyflavone alcohol substrates (Jin et al., 2016). The low expression of *CHI2* in P may lead to the extremely low content of naringin it produces, so it rarely moves downstream to reduce the accumulation of anthocyanin. Meanwhile, the low expression of *F3H*, *DFR1*, and *DFR2* leads to naringin development by the DHQ branch, leading to a low accumulation of cyanidin (Cy) so the spathe displays a light pink color. For example, inhibition of anthocyanin synthesis in transgenic tobacco may be related to the downregulation of CHI in

the anthocyanin pathway (Han et al., 2012). Meanwhile, studies have shown that Cy is the main anthocyanin in pink peach (*Prunus persica*), and the expression of *CHI* and *DFR* in light-pink peach is lower than that in dark-pink peach. *CHI* in the pink chrysanthemum (*Chrysanthemum indicum*) is also lower than in the white chrysanthemum (Chen et al., 2012b; Uematsu et al., 2012). In addition, our results showed high levels of *CHI2* expression in F, providing a large number of substrates and leading to the accumulation of Cy, and the development of the red spathe color. Lower *CHI2* expression, on the other hand, reduced the substrate availability, lowering the anthocyanin content, making the spathe lighter in color, and developing the above pink spathe in the process.

Anthocyanins were not detected in white or yellow calla lily spathes (X. Lei et al., 2017). This may be due to low expression of *DFR1* and *DFR2* that affects anthocyanin synthesis, resulting in the extremely low anthocyanin content and the white spathe. According to existing studies, low expression of *F3H* and *DFR* in carnation (*Dianthus caryophyllus*) flowers leads to decreased anthocyanin accumulation, leading to the white flower phenotype, whereas *DFR* is not expressed in the chrysanthemum white mutant (Feyissa et al., 2009; Totsuka et al., 2018). Thus, low *DFR* expression may hinder anthocyanin synthesis, which is one of the causes of the whitening. Therefore, we hypothesized that the most likely reason for the formation of the white spathe in W was the extremely low expression levels of *DFR1* and *DFR2*. As the anthocyanin synthesis pathway was inhibited and blocked, the synthesis of colorless anthocyanin was reduced, preventing anthocyanin synthesis and resulting in the light and white color.

THE FORMATION OF THE SPATHES MAY BE INFLUENCED BY TRANSCRIPTION FACTOR *bHLH1* AND TRANSPORTER *GST1* IN THE CALLA LILY. According to previous studies in other plants, *Myb*, *bHLH*, and *WD40* form MBW transcriptional activators that act upstream of the anthocyanin biosynthesis pathway genes (Dare et al., 2008). For example, experiments on tobacco have shown that *ANS*, regulated by *bHLH* and *R2R3-Myb* transcription factors in flower color formation, can enhance the flower color of tobacco (Kim et al., 2018). The TT8 site of Arabidopsis encodes a *bHLH* domain protein that regulates the biosynthesis of anthocyanins and procyanidins (Robbins et al., 2003). *bHLH1* was consistently and highly correlated with anthocyanin content and *ANS* expression in B, indicating that *bHLH1* may promote anthocyanin synthesis by regulating *ANS*. The study on the coloration of different radish (*Raphanus sativus*) cultivars found that *RsTT8* (*bHLH*, TF gene) was significantly positively correlated with the expression level of *RsANS* and other genes in the leaves and skins of the root flesh, promoting anthocyanin synthesis (Lim et al., 2017). In addition, a similar phenomenon was reported in the petals of *Centaurea cyanus*, which resulted in anthocyanin accumulation by *CcbHLH1* (Deng et al., 2019). Moreover, the synthesis or accumulation of phytomelanin pigments in the seedcoat of *Ipomoea purpurea* was positively regulated by *bHLH2* (Park et al., 2007). Therefore, we speculate that *bHLH1* may act as a positive regulator of the anthocyanin biosynthesis pathway, promoting anthocyanin synthesis and deepening the color of the spathe.

The coloration of plant floral organs is largely affected by the transport and accumulation of anthocyanins, and GST is one of the transporters responsible for the transport of anthocyanins. Meanwhile, some GSTs also participate in the transport of proanthocyanidins. For example, in *Camelina sativa*, CmGSTF12 is

a functional GST that participates in both anthocyanin and PA accumulation (Wang et al., 2012). To date, although GST has been identified in many plant species, few of them have been identified as anthocyanin-related (Kitamura et al., 2012). The expression of *GST1* in B was highly correlated with the anthocyanin content and the expression of *ANR1* and *ANR2*. Similar studies have found that high expression of *CkmGST3* plays an important role in the accumulation and transport of anthocyanin in cyclamen and peach, and the expression level of AcGST1 in *Actinidia chinensis* is consistent with the degree of anthocyanin accumulation (Cardoso et al., 2012; Cheng et al., 2015; Liu et al., 2019), whereas *GhGSTF8* in the F family of cotton may be related to the transport and accumulation of anthocyanins or PAs (T. Lei et al., 2017). Therefore, we hypothesized that the proteins encoded by *GST1* may be related to the accumulation and transport of both anthocyanins and PAs.

Conclusions

To analyze the effects of genes related to the anthocyanin biosynthesis pathway in the calla lily, the transcriptomic data of the B, P, and W cultivars were analyzed, and qRT-PCR was performed for six different-colored cultivars, including N, F, Y, together with the preceding three. The results showed that the formation of the black spathe is not only dependent on the accumulation of anthocyanins but also related to the interaction between numerous PAs. Meanwhile, *bHLH1* may regulate anthocyanin synthesis, and *GST1* is related to the transport of anthocyanins and PAs. However, the primary reason for the formation of the pink and white spathe is that low expression of *CHI2*, *DFR1*, and *DFR2* leads to inhibition of the anthocyanin synthesis pathway. This study revealed the putative mechanism of color development in the spathes of the calla lily and provides effective genetic resources and a theoretical basis for flower color molecular breeding.

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Supplemental Table 1. Potential flavenoid/anthocyanin biosynthesis pathway genes examined by real-time quantitative PCR (qRT-PCR).

Sequence	Predicted function	Gene name	Primers used for qRT-PCR
Unigene0066235	Chalcone synthase	<i>CHS</i>	TCTGGATCGCCACCCG CCTCTGCCGCATCTCGTCTA
Unigene0049389	Chalcone isomerase	<i>CHI2</i>	GACAAGGTGGCGGAGAACTG GCGTGAAGAGGATGGAGGAAC
Unigene0039079	Flavanone 3-hydroxylase	<i>F3H</i>	AGGGCGGCTTCATCGTC CATGGCCTCGGACAGCA
Unigene0038240	Flavonoid 3'-hydroxylase	<i>F3'H1</i>	GAGGGGCAGTCGTCGGA CAGCAGAGGCACCATAGGC
Unigene0056763		<i>F3'H2</i>	CTGAAGGGTGTATGCCAAATG CTGCCAGTCAAAGGAATGTAGG
Unigene0050009	Dihydroflavonol 4-reductase	<i>DFR1</i>	GAGAAGGCAGCGTGGGACT AGGGCGAGGGCGGAGA
Unigene0047328		<i>DFR2</i>	AGCCCACAATAGATGGAATGC GCGGAGGACGTGAAGACG
Unigene0028183	Anthocyanidin synthase	<i>ANS</i>	CCAAACATCCCTCCAACATACAT GCAGCAGGTCCTCCAAGC
Unigene0040139	Anthocyanidin 3-O-glucosyltransferase	<i>A3GT2</i>	TTGCCAGACCACCTCACCC TCCTTCACCTGCTCCTTCG
Unigene0063644	Anthocyanidin reductase	<i>ANR1</i>	GGACGGGGCTGGTGGT GAAAGATGTGGAGTCAGAGTAGGG
Unigene0033678		<i>ANR2</i>	GGAGAAGGCAGCATGGAAGT CTGAAGGGTGTATCTGGGGTTA
Unigene0041307	Flavonol synthase	<i>FLS</i>	GCAGCTTTTCTTCGTCTTTTGC GTGGTTGCTTATCCTTTTCCCT
Unigene0068785	MYB transcription factors	<i>MYB2</i>	CGTAAGCCATACACCATCACCA CCTCGATCCTTTTCCAGTTCC
Unigene0041675		<i>MYB5</i>	CGCACTGGAAAGAGTTGTCTG CCCCACCTAGCGTGGAGTT
Unigene0044803	WD 40 transcription factors	<i>WD40 1</i>	GTCCAGCCTCATCGTGTCTG GGACGGAGGTGACGGGTT
Unigene0049602	Basic-helix-loop-helix	<i>bHLH1</i>	CCCCTTTTGCTCCCTCG ACCCATCTCGTTTCCTCCTG
Unigene0026291		<i>bHLH5</i>	ATGAAAAGGTGCGGCTAAAGG TGGAAGGAGGCAGCAACAG
Unigene0058553		<i>bHLH6</i>	GTGCCCAATGCCAACAAGT GCTGCTCCGCCTAATCTGC
Unigene0059384	Glutathione S-transferase transporter	<i>GST1</i>	GTCGGTATGGGCGGAAGTG GCTGGTGGTGGAGGTCGG
<i>Cm-Actin</i>			GAAGGAGAACTTGCATGTAGC GTCCATCTGGCAGCTCATAACT
<i>β-tubulin</i>			AGGAAGCTGGCTGTGAATCTAA GAGGGCACGGTATTGTTGG

Supplemental Table 2. Significantly enriched gene ontology (GO) in calla lily of different colors ($P < 0.05$).

GO biological processes	Differentially expressed genes		Corrected <i>P</i>
	with GO annotation	No. with GO annotation	
'Romantic' vs. 'Black Girl' (P vs. B)			
Phenylpropanoid biosynthetic	12	44	0.022106972844406
Flavonoid biosynthetic	11	31	0.003363252660624
Flavone and flavonol biosynthesis	2	3	5.86E-02
'Ventura' vs. 'Black Girl' (W vs. B)			
Phenylpropanoid biosynthetic	12	44	0.011093495527984
Flavonoid biosynthetic	8	31	7.76E-02
Flavone and flavonol biosynthesis	1	3	0.382112864336838
'Ventura' vs. 'Romantic' (W vs. P)			
Phenylpropanoid biosynthetic	10	44	6.92E-02
Flavonoid biosynthetic	13	31	0.000103094357230
Flavone and flavonol biosynthesis	1	3	0.356414550431761

Supplemental Table 3. Kyoto Encyclopedia of Genes and Genomes (KEGG) classification of calla lily.

‘Romantic’ vs. ‘Black Girl’ (P vs. B)				
Pathway	P vs. B_all (843)	P vs. B_up (451)	P vs. B_down (392)	Corrected P
Phenylpropanoid biosynthesis	35	28	7	0.000247724
Flavonoid biosynthesis	13	9	4	0.000158291
Flavone and flavonol biosynthesis	3	0	3	0.05106191
Anthocyanin biosynthesis	1	0	1	0.4890845
‘Ventura’ vs. ‘Black Girl’ (W vs. B)				
Pathway	W vs. B_all (842)	W vs. B_up (490)	W vs. B_down (352)	Corrected P
Phenylpropanoid biosynthesis	31	23	8	0.004457943
Flavonoid biosynthesis	13	11	2	0.000156338
Flavone and flavonol biosynthesis	1	1	0	0.6344041
Anthocyanin biosynthesis	3	0	3	0.01296619
‘Ventura’ vs. ‘Romantic’ (W vs. P)				
Pathway	W vs. P_all (721)	W vs. P_up (393)	W vs. P_down (328)	Corrected P
Phenylpropanoid biosynthesis	32	12	20	0.000145074
Flavonoid biosynthesis	11	7	4	0.000715538
Flavone and flavonol biosynthesis	4	4	0	0.00363813
Anthocyanin biosynthesis	3	1	2	0.008289269

Significantly enriched KEGG biosynthesis of other secondary metabolites in calla lily of different colors ($P < 0.05$).

Supplemental Table 4. Filter cluster graph data of reads per kilobase per million mapped reads (RPKM) in ‘Romantic’ (P), ‘Black girl’ (B), and ‘Ventura’ (W) of calla lily.

Gene No.	Gene Name	P_rpk	Log2(rpkm)	B_rpk	Log2(rpkm)	W_rpk	Log2(rpkm)
<i>CHS</i>	Unigene0066235	17.5252	4.131359003	36.8624	5.204078098	20.8831	4.384263984
<i>CHI1</i>	Unigene0040271	112.7844	6.817423722	116.0702	6.85885381	152.073	7.24862022
<i>CHI2</i>	Unigene0049389	18.4015	4.201751467	42.5071	5.409631931	37.1303	5.214525067
<i>F3H</i>	Unigene0039079	158.3587	7.307052319	117.0474	6.870949078	9.691	3.276645543
<i>F3'H1</i>	Unigene0038240	62.5006	5.965798134	54.7305	5.774273131	18.2316	4.188369272
<i>F3'H2</i>	Unigene0056763	96.0365	6.585510921	125.3603	6.969936727	49.0454	5.616045926
<i>DFR1</i>	Unigene0050009	101.2303	6.661497368	58.4637	5.869469231	25.8132	4.690037095
<i>DFR2</i>	Unigene0047328	63.0861	5.979250261	6.5262	2.706243201	0.9876	-0.018001258
<i>DFR3</i>	Unigene0038442	17.9615	4.166835932	27.0418	4.757119281	62.1207	5.957002182
<i>ANS</i>	Unigene0028183	152.7179	7.254725359	232.2287	7.859402469	56.3211	5.815603606
<i>A3GT2</i>	Unigene0040139	8.1634	3.02917015	12.2006	3.608880193	65.3877	6.030947373
<i>ANR1</i>	Unigene0063644	30.3812	4.92510695	129.6899	7.018922319	28.9715	4.856562477
<i>ANR2</i>	Unigene0033678	15.7607	3.978259707	114.1955	6.835361991	13.7773	3.784221279
<i>FLS</i>	Unigene0041307	38.6458	5.2722397	43.981	5.458808502	31.8907	4.99506386
<i>A3,5GT1</i>	Unigene0076165	208.6819	7.705161664	7.4892	2.904811618	44.0166	5.459975805
<i>A3,5GT2</i>	Unigene0076166	249.1895	7.961099469	40.285	5.33217085	739.9972	9.531376002
<i>Myb1</i>	Unigene0039056	61.7618	5.948642894	68.8724	6.105854046	55.2925	5.789011898
<i>Myb2</i>	Unigene0068785	23.6116	4.561423902	73.998	6.209414373	157.7955	7.301912253
<i>Myb3</i>	Unigene0061741	57.1779	5.837385729	59.4531	5.893680132	52.1185	5.703723658
<i>Myb4</i>	Unigene0046566	43.5255	5.443788966	86.7113	6.438148109	51.4631	5.685466459
<i>Myb5</i>	Unigene0041675	88.2529	6.463571781	159.0144	7.313013609	514.1869	9.006149045
<i>Myb6</i>	Unigene0054464	35.0956	5.133218263	53.5372	5.742469783	34.6468	5.114650205
<i>Myb7</i>	Unigene0054467	41.5874	5.378074587	60.5883	5.920967322	48.6475	5.604293762
<i>Myb8</i>	Unigene0046311	121.3644	6.923201486	150.7524	7.23603716	155.3291	7.279184325
<i>Myb9</i>	Unigene0061026	6.9045	2.787536943	6.8872	2.783917573	43.2012	5.432999482
<i>Myb10</i>	Unigene0050204	37.4206	5.225760786	46.1895	5.529493024	39.1834	5.292170683
<i>Myb11</i>	Unigene0041676	14.5169	3.859661502	27.7812	4.796037012	78.8755	6.30150534
<i>Myb12</i>	Unigene0053473	42.4136	5.406455037	29.3386	4.874728124	12.315	3.622344723
<i>Myb13</i>	Unigene0049779	34.9249	5.12618408	32.9338	5.041497079	52.9179	5.725683906
<i>Myb14</i>	Unigene0049780	15.8726	3.988466562	18.8256	4.234623941	31.3838	4.971948142
<i>Myb15</i>	Unigene0060356	33.4553	5.064162876	43.454	5.44141708	33.869	5.081893486
<i>bHLH1</i>	Unigene0049602	27.2957	4.77060179	128.6541	7.007353624	13.4484	3.749362636
<i>bHLH2</i>	Unigene0049603	6.5445	2.710282974	27.0483	4.757466018	128.4032	7.004537347
<i>bHLH3</i>	Unigene0049604	91.8605	6.521372731	169.0636	7.401422266	158.1652	7.305288398
<i>bHLH4</i>	Unigene0049605	58.7047	5.875404108	52.1139	5.703596319	63.3708	5.985746323
<i>bHLH5</i>	Unigene0049606	13.4229	3.746624492	33.5016	5.066158094	13.2529	3.72823618
<i>bHLH6</i>	Unigene0049607	19.111	4.256331365	70.5515	6.140604851	12.9943	3.699807013
<i>bHLH7</i>	Unigene0049608	32.347	5.015560012	8.7365	3.127055426	47.9405	5.583173051
<i>bHLH8</i>	Unigene0049609	47.3886	5.566468135	8.2063	3.036731896	5.5589	2.474799429
<i>bHLH9</i>	Unigene0049610	8.7531	3.129794053	31.5075	4.977623381	3.866	1.950841638
<i>bHLH10</i>	Unigene0049611	36.6734	5.19666212	31.9309	4.996881312	21.0728	4.397310117
<i>bHLH11</i>	Unigene0049612	31.4025	4.972807514	24.9426	4.640539953	31.1335	4.960395867
<i>bHLH12</i>	Unigene0049613	45.2021	5.498317894	44.887	5.488225773	46.4064	5.536251879
<i>bHLH13</i>	Unigene0049614	39.946	5.319979141	38.4409	5.264570209	44.3967	5.47238054
<i>bHLH14</i>	Unigene0049615	31.8628	4.993801147	26.2918	4.716541011	10.6842	3.417406982
<i>WD40 1</i>	Unigene0044803	92.2956	6.528189967	118.0325	6.883040347	57.3774	5.84241069
<i>WD40 2</i>	Unigene0044804	81.5793	6.350131223	101.2037	6.661118226	82.2297	6.36158766
<i>WD40 3</i>	Unigene0044805	39.1044	5.289259043	47.4618	5.568694911	22.9891	4.522878082
<i>WD40 4</i>	Unigene0044806	38.9902	5.28503965	43.316	5.436828119	40.5885	5.342999119
<i>WD40 5</i>	Unigene0044807	42.1601	5.397806384	33.0766	5.04773904	48.5813	5.602329191
<i>WD40 6</i>	Unigene0044808	17.4808	4.127699305	35.5021	5.14983246	25.9042	4.695114124
<i>WD40 7</i>	Unigene0044809	14.2809	3.836014998	20.055	4.325890061	31.9667	4.998497914
<i>WD40 8</i>	Unigene0044810	23.4084	4.548954422	30.3126	4.921845696	18.7159	4.22619252
<i>WD40 9</i>	Unigene0044811	34.9079	5.125481664	37.5051	5.229014884	37.1382	5.214831988
<i>WD40 10</i>	Unigene0044812	21.8324	4.448398827	34.2471	5.097909923	35.7299	5.159059971
<i>GST1</i>	Unigene0059834	17.4865	4.128169651	183.8009	7.522000021	29.0071	4.858334163

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Supplemental Table 4. (Continued) Filter cluster graph data of reads per kilobase per million mapped reads (RPKM) in ‘Romantic’ (P), ‘Black girl’ (B), and ‘Ventura’ (W) of calla lily.

Gene No.	Gene Name	P_rpk	Log2(rpkm)	B_rpk	Log2(rpkm)	W_rpk	Log2(rpkm)
<i>GST2</i>	Unigene0067732	121.2352	6.921664828	105.099	6.715605132	128.1919	7.002161296
<i>GST3</i>	Unigene0059835	39.9164	5.318909707	190.6335	7.574657856	77.8184	6.282039413
<i>GST4</i>	Unigene0047697	23.607	4.561142809	18.3615	4.198612016	21.2513	4.409479192
<i>GST5</i>	Unigene0021720	39.3798	5.299383879	11.045	3.465321514	46.473	5.538320874
<i>GST6</i>	Unigene0041543	49.3833	5.625951342	8.8692	3.14880398	36.9224	5.206424428
<i>GST7</i>	Unigene0047261	33.8453	5.0808836	33.4209	5.06267868	19.2249	4.264904189
<i>GST8</i>	Unigene0070342	41.4751	5.374173555	39.0238	5.286282364	36.7147	5.198285906