

Transcriptome Analysis Revealed The Mechanism of Exogenous ABA Increasing Anthocyanins in Blueberry Fruit During Veraison

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Abstract

Background: Blueberry (*Vaccinium Spp*) is a popular healthy fruit all over the world. The health value of blueberry is mainly due to the fact that blueberry is rich in anthocyanins, which have a strong antioxidant capacity. However, due to the fact that blueberry is a non model plant, little is known about the structural genes and regulatory genes involved in the anthocyanin synthesis of blueberries. Previous studies have found that spraying abscisic acid at the late green stage of blueberry fruit can increase the content of anthocyanins. Based on the former results, the anthocyanin accumulation process of blueberry can be divided into six stages from late green stage to mature stage to analyze the anthocyanin synthesis mechanism. In order to identify the important genes in the anthocyanin synthesis process of blueberry, the transcriptome analysis was conducted to explore the key genes in blueberry anthocyanin synthesis process.

Results: The results showed that ABA could increase the anthocyanin content of blueberry fruits during the veraison. The effect of ABA on blueberry fruit development was systematically analyzed by KEGG and GO. All structural genes and transcription factors (MYB, bHLH and WD40) involved in anthocyanin pathway were identified and their spatiotemporal expression patterns were analyzed. The expression of *CHS*, *CHI*, *DFR* and *LDOX / ANS* in ABA treated fruits was higher in S5-S6, which was consistent with the change of anthocyanins in fruits. In general, six MYB transcription factors, one bHLH transcription factor and four WD40 transcription factors under treatment were found to have significant changes in transcripts during fruit ripening.

Conclusions: Our results suggest that *VcMYBA* should play a major role in the regulation of anthocyanin synthesis in ABA signaling. This result preliminarily explained the mechanism of ABA increasing anthocyanin content and improves the efficiency of industrial use of blueberry anthocyanins.

Background

Blueberry belongs to the genus *Vaccinium*, and the main cultivated species are northern highbush blueberry plants (*Vaccinium corymbosum* L.), southern highbush blueberry (primarily *Vaccinium corymbosum* L.), lowbush blueberry (*Vaccinium angustifolium* Aiton) and rabbiteye blueberry (*Vaccinium ashei* Reade) [1]. Blueberry fruit is one of the most famous healthy fruits in the world. This is mainly due to the blueberry contains a variety of phytonutrients, the most representative of which is anthocyanins [2]. With the in-depth study of blueberry anthocyanins, a growing number of clinical and animal experiments have proved that blueberry anthocyanins can effectively alleviate obesity [3] and cardiovascular disease [4], and anti type 2 diabetes [5] and cancer [6]. Therefore, the content and variety of anthocyanins is one of the important characters of blueberry fruit, and it is also the direction of breeding.

The structural genes of anthocyanin pathway in plants are well understood, and the important functions of *CHS* [7], *CHI* [8], *DFR* [9], *ANS* [10] and *UFGT* [11] have also been verified. At present, three kinds of transcription factor MYB, bHLH and WD40 have been found to play a major regulatory role in the anthocyanin pathway [12]. They form MBW complex and directly regulate the expression of structural genes, among which MYB transcription factor plays a major role [13]. In *Arabidopsis thaliana*, the expressions of *AtMYB75*, *AtMYB90* [14], *AtMYB113* and *AtMYB114* [15] were positively correlated with the change of anthocyanins content. Similarly, *GL3* and *EGL3* [16] of bHLH family identified in *Arabidopsis thaliana* also showed positive correlation. The loss of *AtTTG1* in WD40 family can affect the expression of *DFR* and other anthocyanin synthesis genes [15]. However, there are few studies on structural genes and transcription factors regulating anthocyanin synthesis in blueberry. Only one MYB transcription factor *VcMYBA* has been found to activate anthocyanin synthesis by activating the promoter of *DFR* [17].

Exogenous ABA can increase the anthocyanin content of non-climacteric fruits such as strawberry [18–19] and grape [20]. Northern highbush blueberry with 1000 mg/L exogenous ABA application were found that the coloration was accelerating and the anthocyanins content increased during the 12 days after treatment [21]. Therefore, the discovery that ABA treatment increased the total anthocyanin content of blueberry fruits can be used as a point of penetration to study the mechanism of anthocyanin synthesis. With the development of high-throughput sequencing, transcriptome has been widely used to study metabolic pathways and key genes. The genome of highbush blueberry (*Vaccinium corymbosum*) were assembled, which could provide a good reference for transcriptome [22]. The mechanism of exogenous ABA on grape berry ripening at 22 and 44 hours was systematic illustrated by RNA-seq [23]. Similarly, transcriptome can be used to systematically study the synthesis mechanism and explore the key regulatory genes in blueberry.

In this study, blueberry fruit was divided into six stages from late green to mature. We systematically analyzed the effect of ABA on the whole transcripts during fruit ripening, especially on anthocyanin pathway. Combined with previous studies on anthocyanin biosynthesis in plants, key transcription factors involved in anthocyanin biosynthesis were identified and their expression patterns were analyzed. Among the key transcription factors, the expression of *VcMYBA* was consistent with that of anthocyanins, indicating that *VcMYBA* plays a role in ABA pathway and is more likely to be related to the high expression of anthocyanins under stress conditions.

Results

Fruit colouration and total anthocyanins content

The developmental stage in cv 'Brightwell' from ripening initiation to mature is divided into six stages (Fig. 1A) by fruit color (Stage 1, the whole fruit is green; Stage 2, the top turn red; Stage 3, the side turn red; Stage 4, the whole fruit is red; Stage 5, the whole fruit turn purple; Stage 6, the whole fruit is purple) and size (Table 1). Application of 1000 mg/ml ABA resulted a significant acceleration of fruit colouration (Fig. 1B). From S3 to S4, the a^* value reflects the top, side and bottom of 1000 mg/ml ABA group turns red earlier than 500 and 0 ABA group. In S5, the top and bottom turns green earlier. From S5 to S6, the b^* value reflects the side and bottom of 1000 mg/ml ABA group turns blue earlier than 500 and 0 ABA group. From value of a^* and b^* , there are no notable difference in three groups in S6. Anthocyanins accumulation is consistent with the change of fruit colouration (Fig. 1C). In S1-S3 stage, almost no anthocyanins were detected. In S4 stage, although the average anthocyanin content of 1000 mg / ml treatment was higher than that of the control group, there was no statistically significant difference. In S5 and S6, total anthocyanins content of 1000 mg/ml ABA treatment is obviously higher than the 0 and 500 mg/ml treatment. From S5 to S6, the anthocyanin content of 0 mg/ml treatment group is nearly doubled and was still lower than that in the 1000 mg / ml group.

Assembly and annotation of the transcriptome

The fruits in Stage1-6 with 0 and 1000 mg/ml ABA treatment were further transcriptome analysis. The raw bases of each sample varied from 7.04 G to 10.91 G with 0.03% error ratio and 46% GC content. In total, 2218553864 clean reads and 2176923110 raw reads were obtained. The raw reads of 12 samples with three biological repeats were uploaded the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (All samples and corresponding explanations can be found in PRJNA664011). For each sample, 87.43%-89.19% of the reads were mapped to the highbush blueberry genome. About 87% of reads were mapped to exonic region, 7% of reads were mapped to intergenic region and 6% of reads were mapped to intronic region. The clean reads were assembled into 128559 unigenes. 88532 unigenes were annotated by Swiss-Prot database, accounting for 68.86% of the total.

Profiling of the transcriptome

The principal component analysis (PCA) was used to analyze the overall variation of transcripts among 32 samples. The 3D plot shows The three repetitions are well clustered together, and the differences between the sample points are obvious (Fig. 2A). The FPKM value of gene in ABA treated group was compared with that in control group. $\log_2(\text{fold change})$ greater than 1.3 or less than -1.3 indicates up-regulation and down regulation of gene expression, respectively. In S2 period, the number of genes with significant changes was about twice as much as that of the control. The number of differentially expressed genes decreased sharply in S3, and then gradually increased in S4-S6 stage (Fig. 2B). Volcano maps were used to represent the overall changes in gene expression between the two groups (Fig. 3C). The results showed that the number of genes with high expression diversity decreased continuously from S1 to S6.

The total amount of anthocyanins in ABA treatment group was higher than that in control group from S4, so the transcripts of S4 were functionally categorized and analyzed by GO and KEGG database (Fig. 4). Both databases Results from both databases showed that the transcripts of the changes were concentrated in metabolic pathways.

Structural genes involved in anthocyanin synthesis

Transcripts of all key enzymes involved in anthocyanin pathway were analyzed (Fig. 4). Green indicates that the expression of transcripts in ABA treated group is lower than that in control group, while red is the opposite. In the whole process, the transcripts of CHS and CHI, two important enzymes in the upstream, all showed consistent down-regulation in S1 and S2, and increased in S5 and S6. Another key enzymes LDOX/ANS showed similar expression pattern and was highly expressed in S3 and S4. The transcripts of the three enzymes F3H, F3'H and F3'5'h on different branches showed different expression in six stages. Similarly, the expression of UFGT transcripts was inconsistent. Although the transcripts of DFR were not consistent to some extent in six stages, they were generally high expression after S3. In general, the transcript expression patterns of CHS, CHI, DFR and LDOX were consistent with the changes of anthocyanins content in fruits.

Identification of key transcription factors involved in anthocyanin synthesis

The change trend of several key structural genes is the same, which indicates that the expression of transcription factors may affect the expression of downstream genes. The published transcription factors from different species were used to construct phylogenetic tree with homologous transcription factors in blueberry. According to the classification of MYB transcription factors [24] and the functions of MYB transcription factors, MYB transcription factors involved in anthocyanin synthesis of blueberry were identified (Fig. 5). Five blueberry MYB transcripts belong to the branch of SG VIII-e2k, and the expression of MYB transcription factors in this branch has been proved to promote anthocyanin synthesis. A blueberry transcript belonging to the SG V δ branch that promotes anthocyanin synthesis was also identified. For the negative regulated SG VIII-E e1 β branch, two transcripts were identified. For bHLH and WD40 transcription factors involved in anthocyanin regulation, four homologous genes were identified (Fig S1 and S2).

Spatiotemporal expression analysis of key transcription factors

Among the identified transcription factors, six MYB transcription factors, one bHLH transcription factor and four WD40 transcription factors showed significant differences in the specific stages (Fig. 6). The expression trend of these transcription factors was verified by qRT-PCR (Fig S3). The expression of two MYB transcription factor in SG VIII-e2k branch was positively correlated with anthocyanin content. Two homologous negative regulatory MYB transcription factors showed up-regulated and down-regulated differential expression in S1. The transcript belonging to SG V δ Branch showed a high increase from S4 to S6, which was earlier than the change of anthocyanin content. The bHLH transcript homologous to *AtGL3* was down expressed in S1-S2, but highly expressed in S4-S5. There was no correlation between the expression of the four WD40 transcripts. The results showed that transcripts belonging to SG VIII-e2k Branch and homologous to *VcMYBA* were most likely related to the increase of anthocyanins.

Discussion

It was reported in many literatures that exogenous ABA could increase anthocyanin content in non-climacteric fruits [18–20]. The anthocyanin content of plants will also increase under many abiotic stresses such as drought [25], low temperature [26] and nitrogen deficiency [27]. The response of plants to these stresses is also related to ABA signaling pathway. For blueberries, previous studies only focused on the 12 days after ABA application at the late green fruit stage [21]. For the mechanism of ABA increasing anthocyanin content, only the transcriptome analysis of 24 h and 48 h after ABA treatment was carried out [23]. It takes about 35 days for the late green fruits to ripen, and the key genes involved in this process need to be studied in detail. Therefore, we divided blueberry fruit into six stages from late green to mature, and analyzed the changes of gene expression in the whole process in detail. The results of transcriptome analysis showed that the expression of structural genes involved in anthocyanin synthesis was inconsistent. In ABA treated fruits, the transcripts of CHS, CHI, DFR and LDOX / ANS, which were proved to be the key structural genes in anthocyanin biosynthesis, were generally low expressed in S1-S2 and highly expressed in S5-S6. Combined with previous studies on transcription factors upstream of these structural genes [15], we speculate that MBW complex is involved in the anthocyanin regulation of ABA pathway. The homologous genes of MBW transcription factors published in important plants were identified by database annotation and phylogenetic tree construction in blueberry. Two *VcMYBA* (*AtMYB75/90* homologous gene) copies, one *AtMYB56*

homologous copy and one *AtGL3* homologous copy were highly correlated with anthocyanin changes. Overexpression of *VcMYBA* [17] and *AtMYB75/90* [15] can enhance anthocyanin synthesis. In addition, *DFR* and *CHS* were also proved to be downstream of *AtMYB75/90* and *DFR* was on the downstream of *VcMYBA*. Our results are consistent with previous studies. The high expression of *VcMYBA* in S5-S6 is consistent with the change of anthocyanins, and the same structural genes *DFR* and *CHS* are highly expressed. *Atmyb56* needs sucrose induction and its anthocyanin increasing effect is relatively low [28]. Combined with the homologous gene of *AtMYB56* in blueberry, it began to be highly expressed in S3, suggesting that the gene did not play a major role. *AtGL3* can enhance the downstream anthocyanin expression by interacting with *AtMYB75/90* [14]. This finding preliminarily explained the mechanism of ABA increasing anthocyanin content in non-climacteric fruits, and broadened the pathway of MYB and bHLH in response to ABA. As ABA is a stress response hormone, this finding also provides a certain reference for explaining the stress-induced increase of anthocyanin content in plants. This result can provide basis and new target for blueberry anthocyanin breeding.

Conclusion

Application of exogenous ABA in the late green period of blueberry fruit resulted in the increase of anthocyanins. The most direct reason for this result is that *CHS*, *CHI*, *DFR* and *LDOX/ANS* are highly expressed in S5-S6. Two *VcMYBA* (*AtMYB75/90* homologous gene) copies, one *AtMYB56* homologous copy and one *AtGL3* homologous copy were found in the upstream of the above genes, and the changes were positively correlated. Among these transcription factors, two copies of *VcMYBA* had the greatest correlation with the high expression of anthocyanins, so they were supposed to be the main influencing factors. This discovery broadens the understanding of the function of *VcMYBA* and the regulation of anthocyanin synthesis pathway in blueberry.

Materials And Methods

Plant materials and ABA treatments

Four years old rabbiteye blueberry 'Brightwell' were grown in experimental base of Baima district, Nanjing city, China. Blueberries of same size and growing condition were selected to do the treatment. Each treatment was arranged in a randomised complete block design with 6 replications and each block replication contains 6 shrubs.

The (+)-Abscisic Acid (Purity 95%, Coolaber company, China) was dissolved in double distilled H₂O containing 5% (v/v) ethanol and 0.1% Tween 80. When most of blueberries fruits were in a growing stage of 'green mature' and the top of the fruit at the top of the branch has just begun to turn red, 0, 500 and 1000 mg L⁻¹ ABA solutions were sprayed on fruit clusters. Tiny sprayer were used to spray the ABA solutions on the peels until the peels are wetted. The leaves and branches were carefully avoided.

In different development stage of fruits, 30 fruits were immediately frozen in liquid nitrogen and stored at -80°C for later experiments.

Physiological characterization

The color of fruit peel

Fruit peel color was measured by colorimeter (Ci64, X-Rite, US) and shown by the International Commission on Illumination a* and b* colour space coordinates [29]. The a* value is negative for green and positive for red and the b* value is negative for blue and positive for yellow, both of the values range from -100 to 100. Due to the coloration of rabbiteye fruits starts from top to bottom, the top, side and bottom of fruit peel were separately measured in the same stage.

The total anthocyanins content

The total anthocyanins content were determined by the double pH differential method [30]: absorbance of the extract was measured at 510 and 700 nm in buffers at pH 1.0 (hydrochloric acid-potassium chloride, 0.2 M) and 4.5 (acetate acid-sodium acetate, 0.2 M). Total anthocyanins content was calculated using a molar extinction coefficient of 29,600 (cyanidin-3-glucoside) and absorbance of $A = [(A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}]$.

Transcriptome analysis

RNA preparation and sequencing

Total RNA was extracted from whole fruits using Trizol reagent (Invitrogen, Carlsbad, CA). The RNA integrity was analyzed on agarose gel. RNA concentration and integrity were measured by Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA) and RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system respectively. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. Then, the libraries were sequenced on an Illumina HiSeq platform and 150 bp paired-end reads were generated.

Reads mapping to the reference genome

Clean reads were obtained by in-house perl scripts that remove reads containing adapter, reads containing poly-N and low quality reads from raw reads. Reference genome and gene model annotation files of *Vaccinium corymbosum* were downloaded from Giga science (<http://gigadb.org/dataset/100537>). Paired-end clean reads were aligned to the reference genome using STAR (v2.5.1b) by the method of Maximal Mappable Prefix. The function of the transcript was annotated by UniProt database (<https://www.uniprot.org/>).

Differential expression analysis

HTSeq v0.6.0 was used to count the reads numbers mapped to each gene and FPKM (fragments per kilobase per million reads) was calculated based on the length of the gene and reads count mapped to this gene. Then, differential expression analysis of two groups was performed using the DESeq2 R package (1.10.1). Genes with P-value using Benjamini and Hochberg's approach were assigned as differentially expressed. Enrichment analysis of differentially expressed genes of physiological processes was carried out by KEGG (<http://www.genome.jp/kegg/>) and GO (clusterProfiler R package).

Construction of phylogenetic trees

The maximum likelihood phylogenetic trees was constructed by by IQ-tree [31]. The optimal alternative model is selected after calculation. The bootstrap value is 1000. The following sequence was downloaded from GenBank accessions:

MYB: *AtMYB75*(NC_003070.9), *AtMYB113*(NC_003070.9), *AtMYB90*(NC_003070.9), *AtMYB114*(NC_003070.9), *PhAN2*(AAF66727.1), *PhPHZ*(ADQ00388.1), *PhDPL* *VvMYBA1*(BAD18977), *VvMYBA2*(BAD18978), *MdMYB10*(ACQ45201), *MdMYBA*(NC_041797.1), *VcMYBA*(MH105054), *AcMYB10*(PSS35990), *AcMYB110*(AHY00: *AtMYB4*(At4g38620), *PhMYB4*(ADX33331.1), *PhMYB27*(AHX24372), *VvMYBC2-L2*(ACX50288.2), *VuMYBC2*(AKR80571), *VvMYBC2* *L1*(AFX64995.1), *AtMYB12*(ABB03913), *SIMYB12*(ACB46530.1), *VvMYBF1*(ACV81697), *AtMYB123*(CAC40021), *VvMYBPA2*(ACK56131.1), *VcMYB17*(ALP43798.1) *MdMYB11*(NC_041797.1), *MdMYB9*(NC_041796.1), *VuMYBPA1*(AKC94840.1), *VvMYBPA1*(CAJ90831.1), *AtMYB5*(NP_187963.1), *VvMYB5b*(AAX51291), *AtMYB56*(AT5G17800), *MdMYB1*(NC_041789.1).

bHLH: *AtEGL3*(AT1G63650), *AtGL3*(AT1G11130), *AmDELILA*(Uniprot:Q38736), *PhJAF13*(Uniprot:O64908), *AtTT8*(AT4G09820), *MdbHLH3*(ADL36597.1), *PhAN1*(FJ227329.1), *VvbHLH1*(Uniprot:A0A438K127), *MdbHLH33*(EI011581.1).

WD40: *AtTTG1*(AT5G24520), *MdTTG1*(GU173814.1), *PhAN11*(Uniprot:O24514), *VvWDR1*(Uniprot:Q19N39), *VvWDR2*(Uniprot:Q19N38).

Quantitative real-time PCR (qRT-PCR) analysis

Eleven transcription factors with altered expression levels were selected for qRT-PCR verification. The specific primers for 11 genes are shown in Table S3. Expression level is relative to the housekeeping gene *Actin* of highbush blueberry [32]. qPCR was conducted using the TB Green® Fast qPCR Mix (Takara, Japan) according to manufacturer's requirements. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression [33]. The experiment carried out three biological repetitions.

Abbreviations

CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3'5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, flavonoid-3-O-glucosyltransferase

Declarations

Declarations of interest

The authors declare that they have no conflict of interest.

Author contributions

TH and WL designed the experiments. TH and ZY performed the experiments. TH analysed the data. WL and WW wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

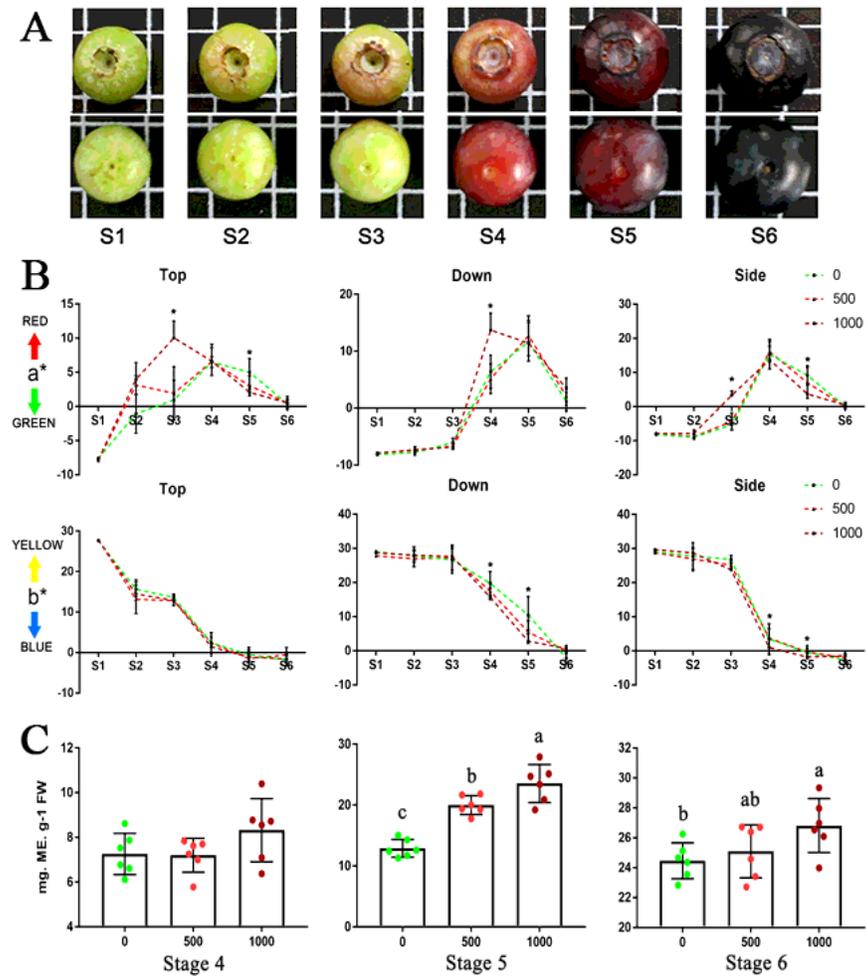


Figure 1

Ripening progress of blueberry (cv 'Brightwell') and colouration and anthocyanins content of fruits under 0, 500 and 1000 mg/ml ABA treatment. (A) The ripening progress is divided into six stages. (B) The color of top, side and bottom of fruits from S1 to S6. (C) Total anthocyanins content form S4 to S6.

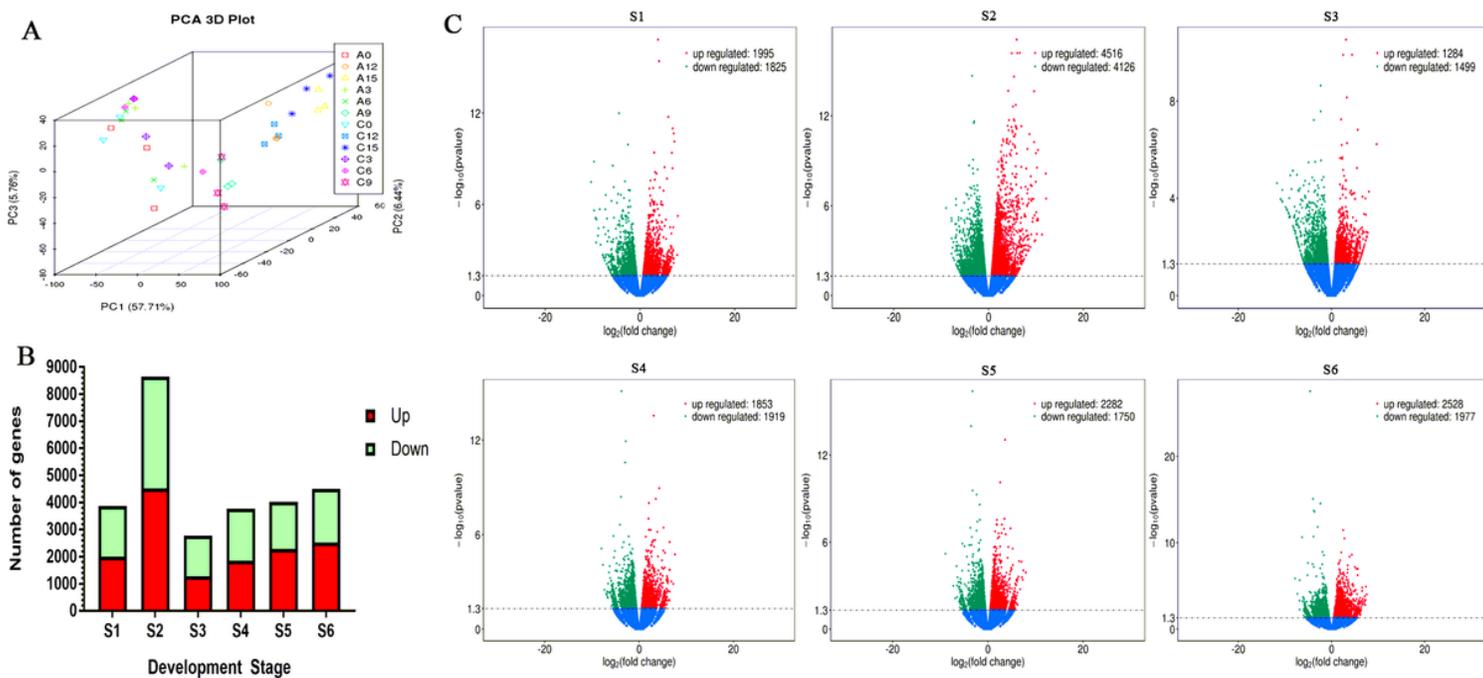
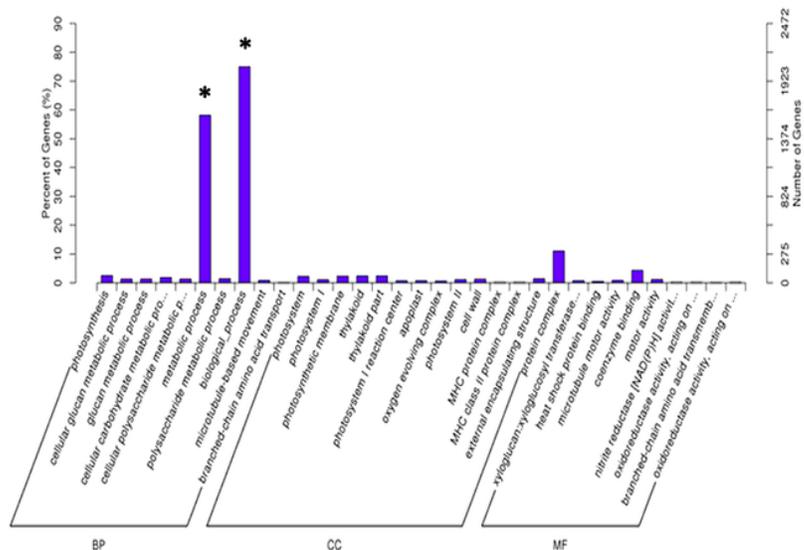


Figure 2

Profiling of transcripts difference between the two groups in six stages. (A) Principal component analysis of each sample. A0-15 represents ABA treatment S1-S6, c0-15 represents S1-S6 of control group (B) The total number of transcripts significantly up-regulated and down regulated in development stages. (C) Volcano maps show overall differences between treatments.

A Enriched GO Terms



B KEGG pathway

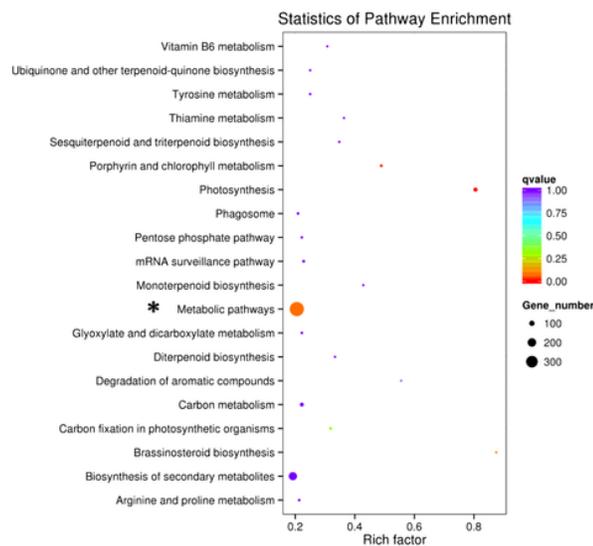


Figure 3

KEGG and go were used to analyze the pathway of significant changes in S4

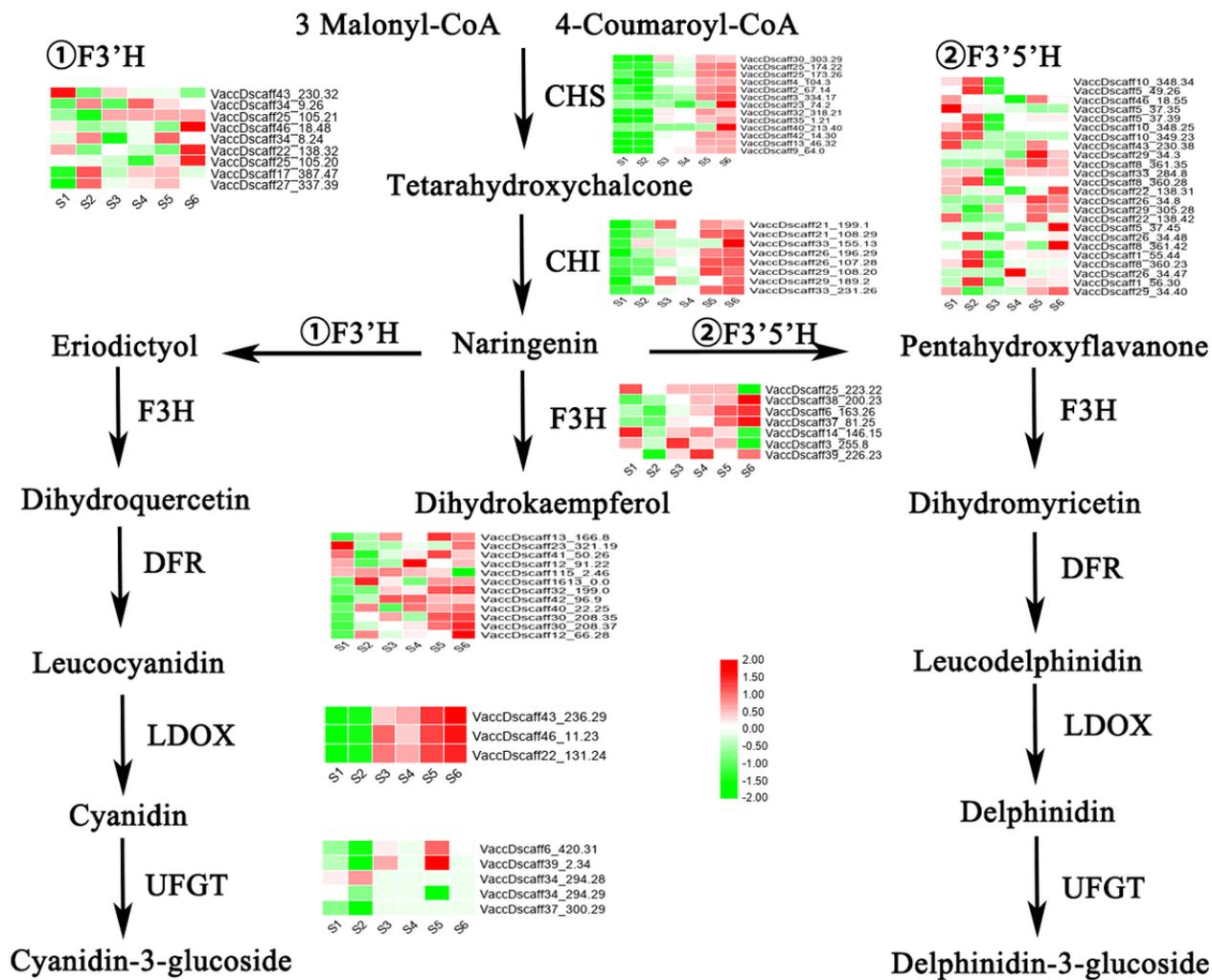


Figure 4

Expression changes of key structural enzymes in anthocyanin pathway

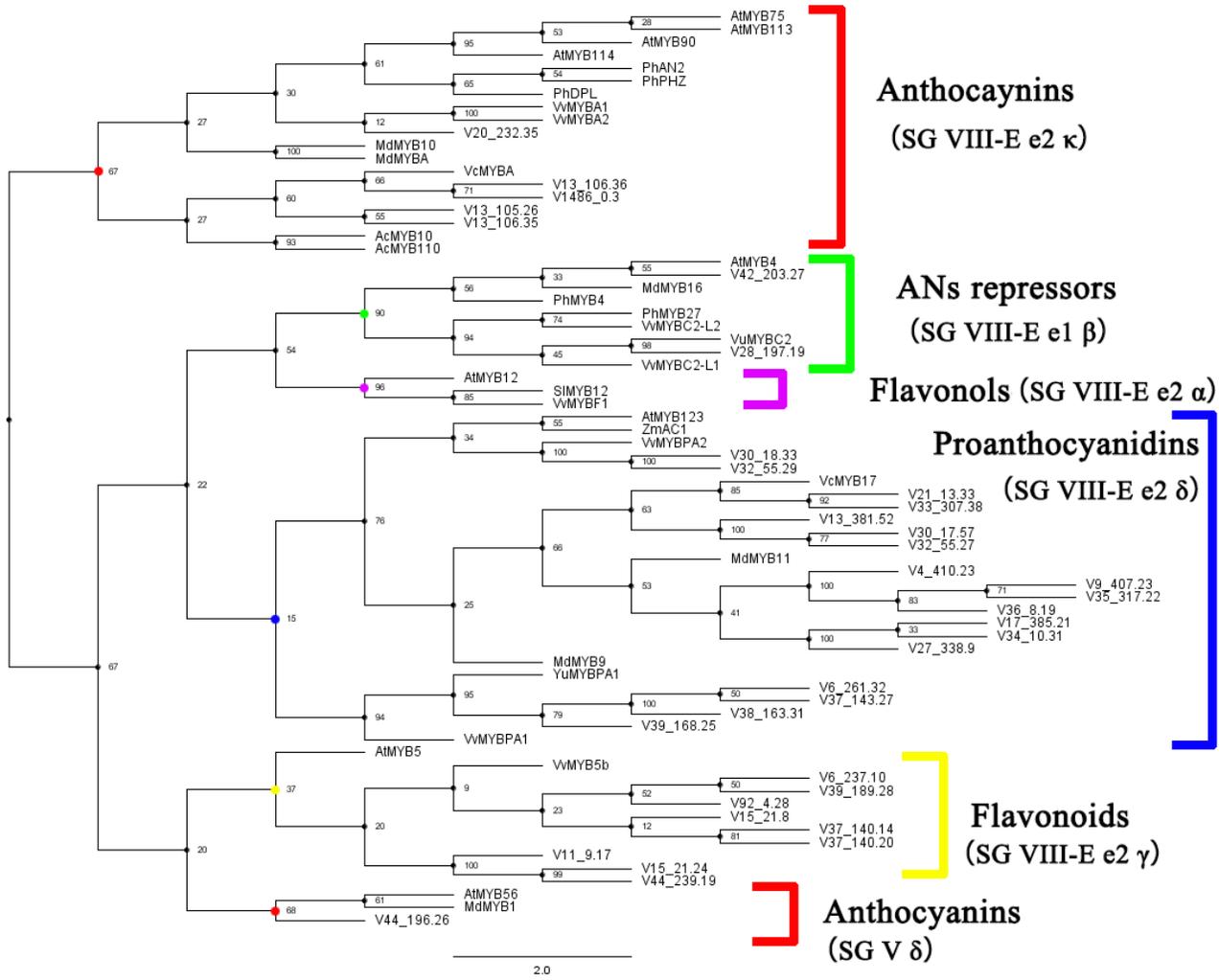


Figure 5

Identification of known MYB transcription factors regulating anthocyanin synthesis in blueberry

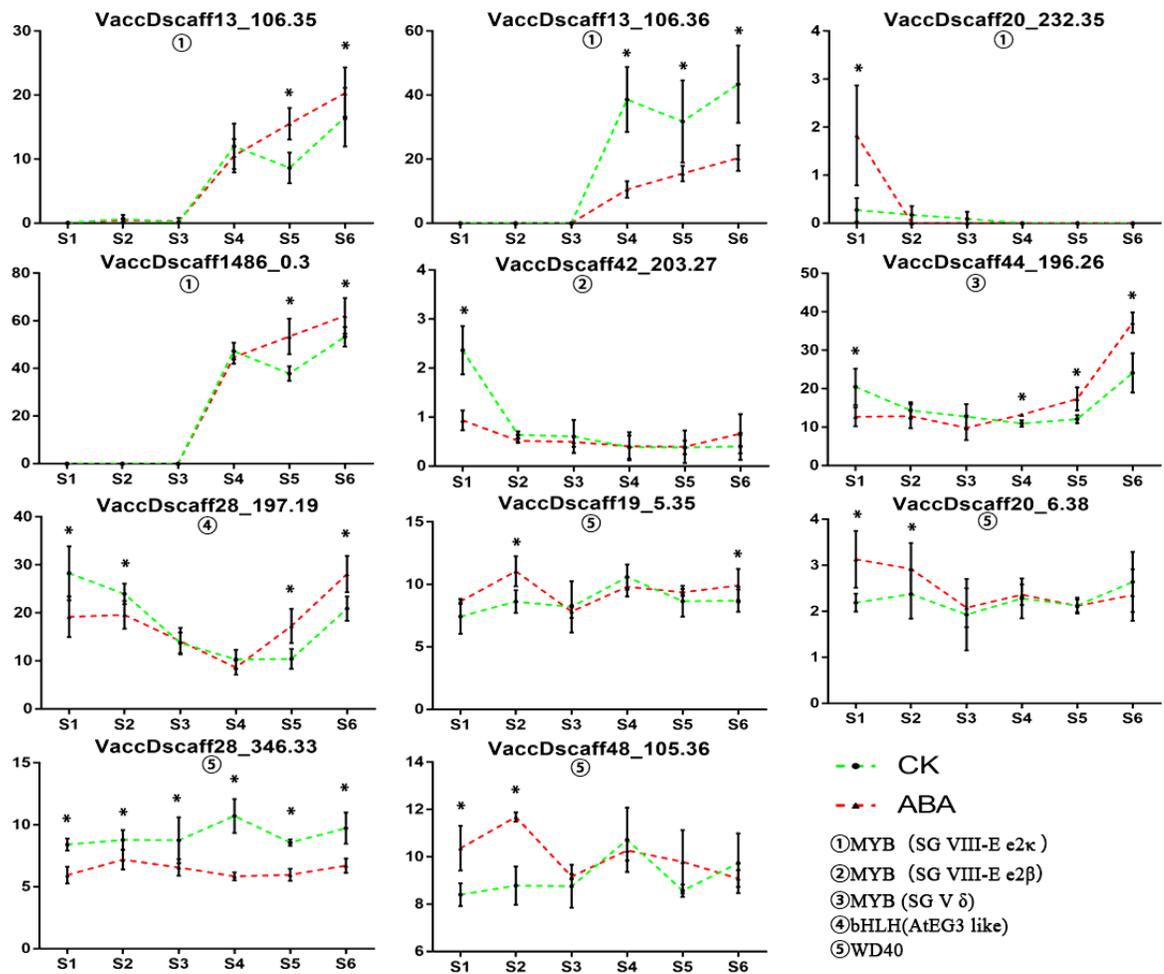


Figure 6

MYB, bHLH and WD40 transcription factors with different expression

Supplementary Files

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- [FigS3.tif](#)
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