INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–0495/2020/24–6–1487–1496
DOI: 10.17957/IJAB/15.1587
http://www.fspublishers.org

Full Length Article

Effects of Abscisic Acid and Sucrose on the Differential Expression of Anthocyanin Biosynthesis Genes in Strawberry

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Received 27 March 2020; Accepted 07 July 2020; Published 10 October 2020

Abstract

Abscisic acid (ABA) and sucrose could promote anthocyanins accumulation. However, the molecular mechanism of ABA, sucrose and interaction of ABA/sucrose in the accumulation of anthocyanins in strawberry fruit is unclear. We applied 95 μM ABA, 100 m*M* sucrose and 95 μM ABA + 100 m*M* sucrose to spray the strawberry fruit at de-greening stage. Subsequently, the treated fruits were collected at 0 and 8 days for RNA sequencing (RNAseq). The result showed that the expression patterns of differentially expressed genes (DEGs) in response to sucrose were similar to that of ABA + sucrose treatment, and ABA was similar to that of control (water), showing that synergistic interaction may occur between ABA and sucrose in promoting anthocyanin accumulation in strawberry fruit. Meanwhile, ABA, sucrose and ABA + sucrose treatments changed transcript level of MYB-bHLH-WD repeat (MBW) complex, *WRKY* and structural genes which are the crucial genes regulating biosynthesis of anthocyanins. The identification and expression analysis of MBW complex, WRKY, structural gene, glutathione S-transferase (GST), auxin response factor (ARF) and auxin responsive protein (Aux/IAA) related to anthocyanin synthesis suggested that up-regulation of *WRKY31* (Cluster-12337.98154), *WRKY71* (Cluster-12337.81062) and 6 *GSTs* (Cluster-12337.70402, Cluster-12337.74163, Cluster-12337.67740, Cluster-12337.80939, Cluster-12337.64226 and Cluster-12337.63035), and down-regulation of *MYB1R1* (Cluster-12337.57122), *bHLH48* (Cluster- 12337.58812), *bHLH51* (Cluster-12337.38826), *bHLH93* (Cluster-12337.103164), *WRKY2* (Cluster-12337.112247), *WRKY53* (Cluster-12337.102451), *WRKY24* (Cluster-12337.25453), *ARF5* (Cluster-12337.73750), *Aux/IAA4* (Cluster-12337.76254) and *Aux/IAA16* (Cluster-12337.73689) contribute to accumulation of anthocyanins in ABA + sucrose or individual treatments. The structural genes of anthocyanin biosynthesis, including *DFR* (Cluster-12337.70165), *UFGT* (Cluster-12337.64199 and Cluster-12337.80836) and *ANS* (Cluster-12337.69226 and cluster-12337.70413), were promising gene candidates for manipulating anthocyanin biosynthesis in strawberries.

Keywords: ABA; Sucrose; Anthocyanins; Gene expression; Transcriptome; Strawberry

Introduction

Anthocyanins are a group of water-soluble flavonoid compounds and widely distributed in various organs of plants, which makes plants blue, purple or red color to perform different biological functions, such as attracting pollinators, seed dispersal, enhancing stress resistance, avoiding UV light damage and regulation of auxin transport (Zhang *et al*. 2014; Lloyd *et al*. 2017). Additionally, anthocyanins have antioxidant properties, help scavenging free radicals, anti-aging, anti-cancer and boost immunity in human body (Holton and Cornish 1995). Therefore, the studies on the mechanism of anthocyanin accumulation are triggering strong interests in scientists. The basic chemical structure of anthocyanins is phenyl-2-benzopyran, which is composed of A and B rings. Generally, there were substituted hydroxyl groups at 3-, 5-, 7-carbon sites of A ring, and the different substituents in the B ring form different kinds of anthocyanins (Fang and Ni 2001). The structure of anthocyanins monomer is not stable and usually coupled with monosaccharide or polysaccharide to form stable glycosides (Tsuda 2012). More than 20 anthocyanins have been found, mainly derived from six anthocyanins: delphinidin (Dp), petunidin (Pt), cyanidin (Cy), pelargonidin (Pg), peonidin (Pn) and malvidin (Mv) (Kong *et al.* 2003). The type and content of anthocyanins determine the color of flower and fruits.

In recent years, a well-studied pathway of anthocyanin synthesis is constructed with a series of enzymes, including anthocyanidin synthase (ANS), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), chalcone isomerase (CHI), dihydroflavonol 4-

To cite this paper: Zhang X, C Ge, M Yang, Y Long, G Hou, Y Liu, Y Luo (2020). Effects of abscisic acid and sucrose on the differential expression of anthocyanin biosynthesis genes in strawberry. *Intl J Agric Biol* 24:1487–1496

reductase (DFR), UDP-glucosyltransferase (UFGT), flavanone 3`-hydroxylase (F3`H), flavanone 3`5`-hydroxylase (F3`5`H), flavonol synthase (FLS), dihydroflavonol-4-reductase (DFR), leucoanthocyanidin reductase (LAR) and anthocyanidinreductase (ANR) (Zhang *et al.* 2014), which contribute to coloration in plant parts. MYB-bHLH-WD repeat (MBW) MBW ternary complex, which comprises one R2R3-MYB protein, one bHLH (helix-loop-helix) protein and one WD-40 protein, regulated the genes coding for anthocyanin biosynthesis enzyme from transcription level in some plant species (Xu *et al.* 2014a). R2R3-MYB is considered as one of the key regulators of anthocyanins biosynthesis. The N-terminal of bHLH has a highly conserved domain, which can bind to MYB to form dimerization, and then to initiate or regulate the expression of genes responsible for anthocyanin biosynthesis (Zimmermann *et al.* 2004). WD40 can promote the interaction between bHLH transcription factors and MYB transcription factors, and enhance the stability of MBW protein complex during anthocyanin synthesis. The MBW complex activates the transcription of late biosynthetic genes (*DFR*, *ANR*, *UFGT*, *LAR* and *ANR*) involved anthocyanin pathway (Liu *et al.* 2018). *ANT1* (MYB) (Mathews 2003), *MdMYBl0* (Espley *et al.* 2007), *PpMYB9*, *PpMYB10* (Zhou *et al.* 2016), *VvMYB5a* (Deluc *et al.* 2006), *FaMYB10* and *FvMYB10* (Lin *et al.* 2010) have been identified and proved to be involved in the synthesis of anthocyanins. It has been reported that the production of pigment in maize (*Zea mays*) needs the existence of bHLH (Ludwig *et al.* 1989). In strawberry, *FvbHLH* promoted anthocyanins accumulation under light by co-regulated with *FvHY5* (Li *et al.* 2020). In apple, the induction of anthocyanins by *MdMYB10* mainly depends on the co-expression of *MdbHLH3* and *MdbHLH33* (Espley *et al.* 2007), and *FvMYB10* and *FvbHLH33* may form a complex to promote transcription of structural genes in strawberry (Lin-Wang *et al.* 2014). More recently, it was reported that the WRKY transcription factors (*TTG2*/*PH3*) cooperated with the MBW complex to regulate anthocyanin accumulation (Gonzalez *et al.* 2016; Verweij *et al.* 2016). *MdWRKY40* was essential for wound-induced anthocyanin accumulation in association with *MdMYB1*.

Except genetic factors, environmental elements, sugar and phytohormones can affect anthocyanin biosynthesis in plant. As an important plant hormone, abscisic acid (ABA) has been shown to regulate plant development and growth, and stress-response signaling (Hirayama and Shinozaki 2007). Recently, it has been shown that ABA regulates the ripening of nonclimateric fruit as a key factor (Coombe 1992; Davies *et al.* 1997; Giovannoni 2001) and enhanced anthocyanin synthesis in grape and strawberry (Jia *et al.* 2013). ABA and sugar undergo extensive crosstalk (Finkelstein and Gibson 2002; Carrari *et al.* 2004). It has been reported that sucrose is involved in ABA signaling pathway and co-regulated fruit growth and ripening related genes expression in grapes (Cakir *et al.* 2003; Jia *et al.* 2017). In fleshy fruits, sugar accumulation and metabolism were affected by ABA (Kobashi *et al.* 1999; Pan *et al.* 2005). Meanwhile, sucrose can also affect the content of ABA in strawberry fruit (Luo *et al.* 2019). Recently, it has been reported that sucrose can regulate strawberry fruit ripening and anthocyanin accumulation by ABA-independent and ABA-dependent pathways (Jia *et al.* 2013). The complexity of the multiple and synergistic signals may meet the need for the orderly development and ripening of fruits. Except ABA, auxin also plays an important role in regulation of anthocyanin accumulation in plants (Tsukasa *et al.* 1994; Daminato *et al.* 2013).

Color is an important index to evaluate the maturity and quality of fruit, which is not only regulated by genetic factors, but also by external factors, such as light, water, temperature, ABA and sucrose (Azuma *et al.* 2011; Jaakola 2013). Changes in environmental factors cause poor fruit color, which affects fruit quality and reduce commodity value. Therefore, it is a convenient and quick approach to take plant growth regulators and sucrose to manipulate the color of fruits.

Strawberry (*Fragaria* × *ananassa*) is a model plant for studying non-climacteric fruit (Li *et al.* 2011; Cherian *et al.* 2014). It has been proven that ABA and sucrose participate in strawberry fruits ripening and coloring (Chai *et al.* 2011), and ABA + sucrose treatment had the greatest effect in our previous study (Luo *et al.* 2019; Ling *et al.* 2019). However, the molecular mechanism of ABA, sucrose and ABA/sucrose interaction in anthocyanin accumulation in strawberry fruit is still unclear. As a typical non-climacteric fruit, strawberry is favored by consumers for its unique flavor, rich nutrients and bright color. Among the fruit quality attributes, bright color may affect consumers' purchasing psychology and decision. Interestingly, our previous results showed that exogenous ABA and sucrose could accelerate the synthesis of anthocyanins during strawberry fruit development and ripening, while not changing the final anthocyanin concentration of full-red strawberry (Ling *et al.* 2019). Therefore, there is a need for further research on how ABA and sucrose affect anthocyanin accumulation in strawberry fruit at molecular level. Based on our previous study, 95 μM ABA, 100 m*M* sucrose and 95 μM ABA + 100 m*M* sucrose were selected to spay strawberry fruit at the de-greening stage. Subsequently, the treated strawberry fruits were used for transcriptome sequencing and analysis and the regulatory and structural genes involved in anthocyanin metabolism were assessed. The objectives of this present study is not only to understand the functions of ABA and sucrose in anthocyanin biosynthesis of strawberry, but also to divulge promising genes candidate for manipulating strawberry anthocyanin biosynthesis, which might have a potential suggestion for improving commodity value and quality of strawberry.

Materials and Methods

Plant materials and treatments

The strawberry (*Fragaria* × *ananassa* cv. Benihoppe) plants were grown in a plastic greenhouse in a farm under natural culture conditions in Chengdu, China. A total of about 500 strawberry plants were selected, and 2000 secondary flowers were tagged. Subsequently, fruits at the de-greening stage (18 days after flowering, DG) were treated with water, 95 μM ABA, 100 m*M* sucrose, or 95 μM ABA+100 m*M* Sucrose, respectively. Based on the coloring difference in field, the fruits of 0 and 8 days after treatment were picked, frozen in liquid nitrogen and then stored at -80℃ for RNA extraction.

RNA extraction and sequencing

Total RNA was extracted from the fruit at 0 and 8 days after treatment by using a RNeasy Plant Mini Kit (Qiagen, Dusseldorf, Germany), and DNA was removed with RNase-Free DNase (Qiagen). Final extracted RNA samples concentration and integrity were determined by Nanodrop 1000 spectrophotometer and Agilent 2100 Bioanalyzer. The samples were named CK0, CK8, ABA8, Scu8 and AS8, respectively. Sequencing of five cDNA libraries (CK0, CK8, ABA8, Scu8 and AS8) was performed using the Illumina HiSeq 2000 platform (Novogene Biotechnology Company, Beijing, China). All analyses were conducted using replicates.

Transcripts assembly and expression quantification

Considering the incompleteness of the genome of cultivated strawberry (*Fragaria* × *ananassa*), all clean reads were *de novo* assembled by Trinity software version 2.4.0 (Grabherr *et al.* 2011). To obtain protein-coding transcripts, the assembled non-redundant transcripts were compared with protein databases including Swiss-Prot (http://www.uniprot.org) and NCBI non-redundant (NR) by using Diamond BLASTx (Buchfink *et al.* 2015). RNA-Seq gene expression levels were quantified and normalized by calculating the fragments per kilobase million (FPKM) values using RSEM (Li and Dewey 2011). Pearson correlation coefficient was analyzed by Minitab 15, which could be denoted by $r_{x,y}$ and calculated as follows:

$$r_{X,Y} = \frac{\sum_{i=1}^{n}(X_i - \overline{X})(Y_i - \overline{Y})}{\sqrt{\sum_{i=1}^{n}(X_i - X)^2 \sum_{i=1}^{n}(Y_i - \overline{Y})^2}}$$

Where n was the number of samples from different experiments and X_i and Y_i were expression profile values of probe sets X and Y in the ith sample, respectively. DESeq2 R package (Love *et al.* 2014) was applied for determining differential expression analysis. Significantly differentially expressed genes (DEGs) were defined as genes with the absolute value of False Discovery Rate (FDR) at < 0.05 and $\log_2$ fold change ($\log_2$ FC) ≥1.

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis

DEGs were subjected to GO and KEGG enrichment analysis implemented by Topgo R software package (Alexa *et al.* 2006) and KOBAS 3.0 software (Xie *et al.* 2011), respectively. The application of R ggplot function visualized the data of enrichment analysis of DEGs. Values for heat maps were created using each of the normalized sample FPKM expression values. Subsequently, TBtools software was used to draw heat map.

Results

Summary of sequencing data quality and gene mapping

In order to fully understand the transcriptional responses of strawberry fruit to ABA and sucrose, the fruit of 0 and 8 days after treatments were selected as time point of RNA-seq, and named as CK0, CK8, ABA8, Suc8 and AS8. The original data were submitted to Sequence Read Archive (SRA) database in NCBI website (Accession ID: PRJNA565646; Link: https://www.ncbi.nlm.nih.gov/sra/PRJNA565646). Five samples were sequenced to obtain over 41 million high-quality clean reads, which accounted for 87.48, 96.39, 96.50, 96.4 and 96.58% of the raw reads, respectively (Table 1). In addition, 65.03, 69.90, 70.90, 71.63 and 71.65% of the five samples (CK0, CK8, ABA8, Suc8 and AS8) sequences were mapped to the strawberry (*Fragaria vesca*) reference genome. The mean GC content in the transcripts was about 47%. More than 96.82 and 92.21% of the reads had a quality score over Q20 and Q30, respectively. These results indicated that the assembly quality of high throughput RNA sequencing was good and the mismatch rate was low.

DEGs and KEGG enrichment

In order to ensure the accuracy and reliability of the expression data, Pearson correlation coefficient was applied to calculate gene expression correlation. According to the data, all correlation coefficients (R^2 values) between biological replicates were larger than those outside biological replicates (Fig. S1). DEGs were analyzed by using Deseq R package (v.1.10.1) based on the negative binomial distribution (Padj < 0.05). The results were shown through the Venn diagram (Fig. 1). There were 346, 623 and 3676 DEGs specific to the ABA, sucrose and ABA + Sucrose treatments, respectively, whereas 1003 DEGs were common to the three different comparisons, suggesting that the gene expression patterns showed different response to ABA, sucrose, and ABA + Sucrose treatments.

Table 1: Summary statistics of the sequencing data

Sample	Raw Reads	Clean Reads	Clean Reads/Raw Reads (%)	Clean Bases	Error Rate (%)	Q20 (%)	Q30 (%)	GC (%)	Total mapped (%)
CK0	47958757	41954921	87.48	6.31G	0.02	96.82	92.21	47.92	65.03
CK8	48498646	46746930	96.39	7.01G	0.01	98.10	95.20	47.06	69.90
ABA8	48357580	46663692	96.50	7.00G	0.01	98.12	95.23	46.94	70.90
Suc8	50404051	48601499	96.42	7.29G	0.01	96.99	92.57	46.81	71.63
AS8	48325528	46675098	96.58	7.00G	0.01	98.10	95.11	46.82	71.65

Q20, Q30: The percentage of bases with a Phred value of >20 or 30, Phred = -10$\log_{10}$(e); CK0, CK8; ABA8, Suc8, AS8 represent three biological replicates for control, ABA, sucrose and ABA + Sucrose treatment, respectively

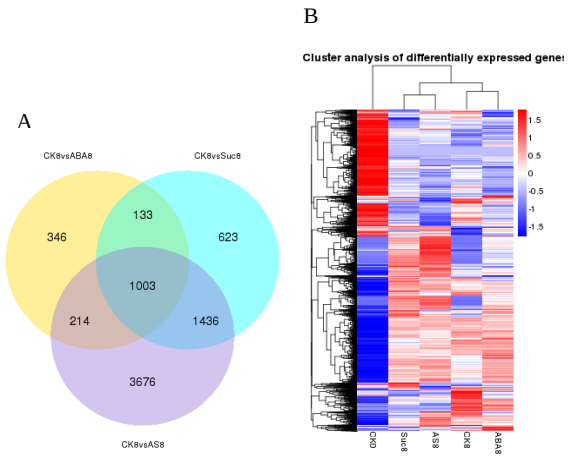

Fig. 1: Differentially expressed genes expression in response to ABA, sucrose, and ABA + Sucrose treatment. (A)Venn diagram showing DEGs distributions; and (B) expression profile clustering. Red and blue colors mean up-expression and down-regulated expression of genes, respectively. CK0, CK8, ABA8, Suc8 and AS8 indicate the 0 day and the 8th samples treated with water, ABA, sucrose and ABA + Sucrose, respectively

Subsequently, we subjected the five samples to hierarchical clustering analysis. The results showed that samples of Suc8 and AS8 clustered together, and ABA8 and CK8 clustered together (Fig. 2B), indicating these genes after sucrose or ABA + sucrose treatment had similar expression profiles, and ABA8 had similar expression patterns to CK8. The potential effects of ABA, sucrose and ABA + Sucrose on the altered biological pathways needed to be investigated. KEGG (http://www.genome.jp/kegg/) enrichment analysis of DEGs was carried out (Fig. 2). A total of 20028 DEGs were mapped to the KEGG database, and the top 20 enriched pathways in differenct treatments were revealed in the present study. In CK8 vs ABA8 (Fig. 2A), "Linoleic acid metabolism", "alpha-Linolenic acid metabolism" and "Flavonoid biosynthesis" were the most enriched KEGG pathway terms, which included 11 DEGs, 19 DEGs and 11 DEGs, respectively. Meanwhile, the "flavonoid biosynthesis", "pantothenate and CoA biosynthesis", and "glutathione metabolism" were enriched in CK8 vs Suc8 (Fig. 2B). In CK8 vs AS8 (Fig. 2C), the most enriched pathway terms were "photosynthesis - antenna proteins", "oxidative phosphorylation", and pyruvate metabolism" (Fig. 2C). Anthocyanins were the main pigment in strawberry fruit coloration. Therefore, we focused on the flavonoid biosynthesis pathway and anthocyanin biosynthesis pathway.

Transcription factors

It has been reported that MYB, bHLH, WD40 and WRKY are critical transcription factors for anthocyanin biosynthesis. In this study, 12 *MYB*, 3 *bHLH* and 12 *WRKY* were identified (Fig. 3). Compared with CK8, ABA treatment significantly changed the expression level of *MYB* and *WRKY*. The expression level of *LHY-like* (Cluster-12337.69059), *MYB44-like* (Cluster-12337.54712), *WRKY40* (cluster-12337.60739), *WRKY24* (cluster-12337.25453), *WRKY11* (cluster-12337.97104) and *WRKY46* (cluster-12337.104131) were significantly down-regulated by 0.99, 1.11, 4.39, 2.62, 2.14 and 2.95 times, respectively. Meanwhile, the expression level of *MYB114-like* (Cluster-12337.67445) and *WRKY71* (Cluster-12337.81062) were up-regulated by 2.02 time and 0.70 time, respectively (Fig. 3A, B).

The number and expression characteristic of *MYB* identified in Suc8 was similar to that in ABA8. However, there was a greater change in gene expression level in Suc8 compared with ABA8. Compared with CK8, the expression level of *WRKY40*, *WRKY11*, *WRKY46* and *WRKY33* were significantly down-regulated by 3.25, 1.86, 2.37 and 2.18 times, and that of *WRKY31* (Cluster-12337.98154) and *WRKY71* were significantly up-regulated by 1.55 times and 2.26 times, respectively. Furthermore, there was no significant difference in the expression of *bHLH* after ABA or sucrose treatment (Fig. 3C).

ABA, sucrose or ABA + sucrose treatments had similar effect on the expression level of *MYB*, but *MYB1R1* (Cluster-12337.57122), *bHLH48* (Cluster-12337.58812), *bHLH51* (Cluster-12337.38826) and *bHLH93* (Cluster-12337.103164) were identified in AS8, the expression level of which was significantly down-regulated compared with CK8. Furthermore, ABA + sucrose treatment significantly reduced the expression level of *WRKY2* (Cluster-12337.112247), *WRKY53* (Cluster-12337.102451) and *WRKY24* (Cluster-12337.25453), and increased the expression of *WRKY31* and *WRKY71*, which was higher in AS8 treatment compared with that of Suc8 treatment. It is interesting to note, *LHY-like* and *MYB44-like* in significantly down-regulated genes, and *MYB114-like* and

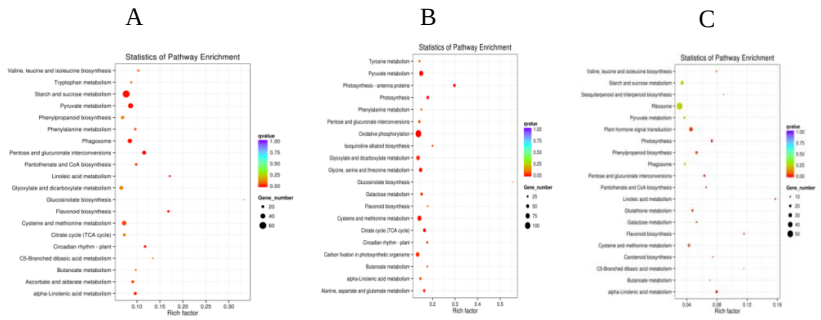

Fig. 2: Top 20 enriched KEGG pathways of DEGs in three pairwise comparisons. (A) CK8 vs ABA8; (B) CK8 vs Suc8; (C) CK8 vs AS8. The vertical axis on the left represents KEGG pathways, the horizontal axis indicates the Rich factor. Low qvalue are shown in red, and high qvalue are depicted in purple. Qvalue < 0.05 are significantly enriched. The size of the spot reflects the number of DEGs, and the color of the spot corresponds to different qvalue ranges. CK8, ABA8, Suc8 and AS8 indicate the 8th samples treated by water, ABA, sucrose and ABA + sucrose, respectively

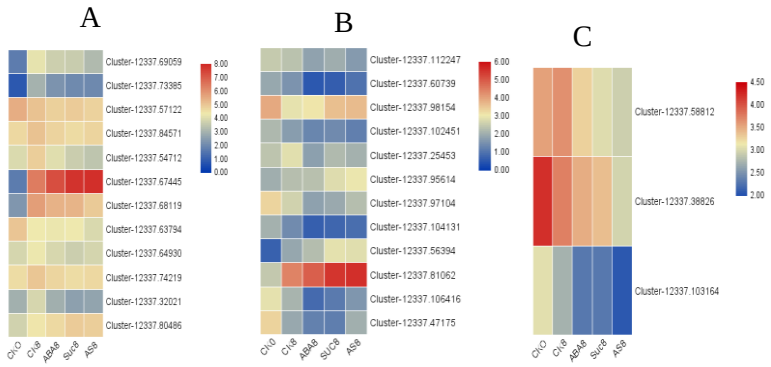

Fig. 3: Expression profile clustering of transcription factors related to anthocyanin biosynthesis. (A) MYB; (B) WRKY; (C) bHLH, basic helix-loop-helix. The color scale at the right represents the re-processed $\log_{10}$ (FPKM+1) using Pheatmap, representing the relative expression level. The expression variance for each gene is indicated by colors ranging from low (blue) to high (red). CK0, CK8, ABA8, Suc8 and AS8 indicates samples of 0 and 8 days after water, ABA, sucrose and ABA + Sucrose treatments, respectively

WKRY71 in significantly up-regulated genes were quite common in ABA8, Suc8 and AS8 treatments (Fig. 3).

Structural genes involved in the anthocyanin pathway

Based on RNA-Seq data, we identified 36 anthocyanin biosynthesis structural genes related to *PAL*, *C4H*, *CHS*, *F3H*, *F3`H*, *F3`5`H*, *DFR*, *FLS*, *UFGT* and *ANR* genes, respectively. Most of *PAL*, *C4H*, *CHS*, *F3H*, *DFR*, *ANR* and *UFGT* were up-regulated with the development of strawberry fruit (Fig. 4). Compared with CK8, most of 'early' flavonoid biosynthetic step genes, such as *CHS*, *CHI* and *F3H*, with enhanced expression levels were up-regulated in ABA8, Suc8 and AS8 treatments: especially in Suc8 and AS8 treatments. Meanwhile, of the 'late' flavonoid biosynthetic steps genes, two members of eight *DFR* (Cluster-12337.70165 and cluster-12337.64510), one member of three *UFGT* (Cluster-12337.64199*)* and all members of *ANS* were significantly up-regulated in ABA8, Suc8 and AS8 treatments, especially in AS8 treatment. Meanwhile, two members of eight *DFR* (Cluster-12337.97275 and Cluster-12337.61538), one member of three *UFGT* (Cluster-12337.80836) and one member of three *ANR* (Cluster-12337.70357) were significantly up-regulated in Suc8 and AS8 treatments, contrasting with their having lower expression level in CK8. Additionally, there was one member of eight *DFR* (Cluster-12337.34755) only in Suc8 treatment and one member of three *ANR* (Cluster-12337.71858) only in AS8 treatments were significantly up-regulated compared with CK8. Moreover, one member of three *UFGT* (Cluster-12337.61103) was significantly inhibited in ABA8 and Suc8 treatments (Fig. 4). Therefore, strong signals for transcripts of *DFR*, *ANS*, *ANR* and *UFGT* were clearly seen in sucrose and ABA + sucrose treatment, which was consistent with the higher level of anthocyanins in strawberry fruit under these treatments in our previous study (Ling *et al.* 2018).

Expression pattern of glutathione S-transferase

Glutathione S-transferase (GST) played an important role in plant primary and secondary metabolism, hormone

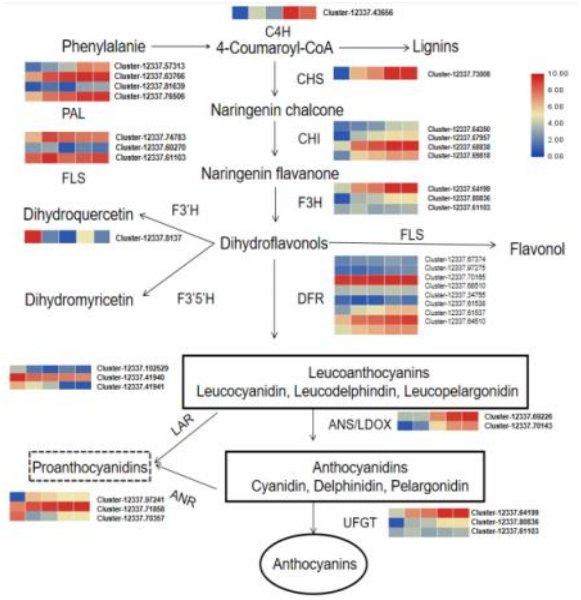

Fig. 4: Effect of ABA, sucrose and ABA + Suc on the expression of genes encoding flavonoid and anthocyanins biosynthetic enzymes in strawberry. The expression pattern of each structural gene in CK0, CK8, ABA8, Suc8 and AS8 isarranged from left to right., CK0, CK8, ABA8, Suc8 and AS8 indicates the samples of 0 and 8 days after water, ABA, sucrose and ABA + Sucrose treatments, respectively. The color ratio represents $\log_{10}$ (FPKM + 1) reprocessed with Pheatmap, representing the relative expression level. The expression of each gene varies from low (blue) to high (red) color. PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate-CoA ligase; C4H, cinnamate 4-hydroxylase; CHS, chalcone synthase; F3`H, flavanone 3`-hydroxylase; F3`5`H, flavanone 3`5`-hydroxylase FLS, flavonol synthase; DFR, dihydroflavonol-4-reductase; ANR, anthocyanidinreductase; LAR, leucoanthocyanidinreductase; UFGT, flavonoid 3-O-glucosyltransferase

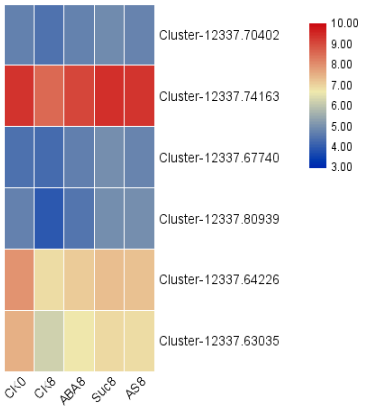

Fig. 5: Effect of ABA, sucrose and ABA + Suc on the expression of genes encoding glutathione S-transferase (GST). The color scale at the right represents the re-processed $\log_{10}$ (FPKM+1) using Pheatmap, representing the relative expression level. The expression variance for each gene is indicated by colors ranging from low (blue) to high (red). CK0, CK8, ABA8, Suc8 and AS8 indicates samples of 0 and 8 days after water, ABA, sucrose and ABA + Sucrose treatments, respectively

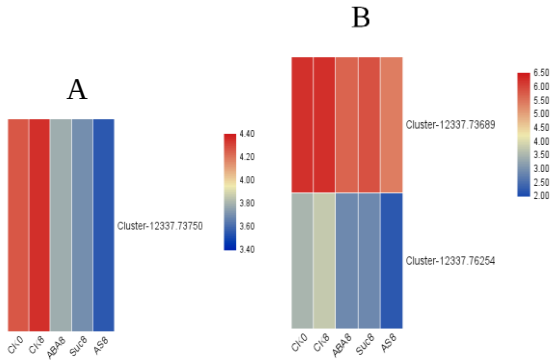

Fig. 6: Effect of ABA, sucrose and ABA + Suc on the expression of genes related to auxin singaling pathway. (A) auxin response factor (ARF); (B) Auxin responsive protein (Aux/IAA). The color scale at the right represents the re-processed log10 (FPKM+1) using Pheatmap, representing the relative expression level. The expression variance for each gene is indicated by colors ranging from low (blue) to high (red). CK0, CK8, ABA8, Suc8 and AS8 indicates samples of 0 and 8 days after water, ABA, sucrose and ABA + Sucrose treatments, respectively

response, oxidative stress and herbicide detoxification. A total of 54 *GSTs* were screened in this study, and the analysis of differential gene expression showed that 6 *GSTs* (Cluster-12337.70402, Cluster-12337.74163, Cluster-12337.67740, Cluster-12337.80939, Cluster-12337.64226 and Cluster-12337.63035) were significantly up-regulated by ABA8, Suc8 and AS8 treatments compared with CK8, especially in Suc8 (Fig. 5), suggesting that GST proteins might be involved in ABA and sucrose induced anthocyanin accumulation.

Auxin signaling transduction related to anthocyanin biosynthesis

Auxin response factor (ARF) and auxin responsive protein (Aux/IAA) are related to auxin signaling transduction, which were involved in anthocyanin accumulation as negative regulators. In this study, 61 *ARFs* and 31 *Aux/IAAs* were screened in the result. Among them, the expression level of *ARF5* (Cluster-12337.73750), *Aux/IAA4* (Cluster-12337.76254) and *Aux/IAA16* (Cluster-12337.73689) was significantly down-regulated in ABA8, Suc8 and AS8 treatments, especially in AS8 treatment (Fig. 6). Other *AFRs* and *Aux/IAAs* showed no significant changes in the three treatments. These results suggested that *AFR5*, *Aux/IAA4* and *Aux/IAA16* might play roles in anthocyanin accumulation.

Discussion

In plants, many studies have demonstrated that anthocyanin

biosynthesis is coordinately regulated by intricate regulatory networks of developmental signals and environmental factors (Zimmermann *et al.* 2004; Hichri *et al.* 2011). However, whether ABA and sucrose promotes anthocyanin biosynthesis remains elusive and exactly how they stimulation promotes anthocyanin biosynthesis remains unclear. In this study, RNA-seq analysis enabled comparative analysis of differential transcriptional genes between different treatments. Approximately 79% of DEGs in AS8 were not found in the DEGs of ABA8 or Suc8 (Fig. 1A), suggesting the synergistic interaction may happen between ABA and sucrose. Meanwhile, the DEGs of ABA, sucrose and ABA + sucrose treatment were all enriched in flavonoid biosynthesis pathways (Fig. 2), which is related to anthocyanin biosynthesis, suggesting that ABA and sucrose treatment affect anthocyanin biosynthesis of strawberry fruit at the molecular level.

It is well known that MBW complex regulates the flavonoid pigment biosynthetic genes in many plants whereby its diversity of combination and interaction with related genes (Xu *et al.* 2015) and is modulated by light, sugar and hormones (Das *et al.* 2012). The MYB transcription factors have been shown to interact closely with bHLH and WD40 to regulate anthocyanin synthesis (de Vetten *et al.* 1997; Nesi *et al.* 2001; Zimmermann *et al.* 2004; Hichri *et al.* 2011). However, the role of bHLH and WD40 on anthocyanin biosynthesis and accumulation in strawberry fruit under ABA regulation is unknown. Meanwhile, it has been reported that ABA and sucrose mainly affects anthocyanin level via regulation of the transcription levels of *AtWRKY40 and AtWRKY60* (Liu *et al.* 2012) as well as *AtMYB44* (Li *et al.* 2014) in *Arabidopsis.* In our study, most of the *MYB*, *bHLH* and *WRKY* were down-regulated after ABA, sucrose and ABA + Sucrose treatments, suggesting that these genes may act as negative regulatory factors for the regulation of anthocyanin synthesis. Zhang *et al.* (2018) reported that blue light promotes anthocyanin accumulation of strawberry fruit, while most of *MYB*, *bHLH* and *WRKY* were inhibited at the level of transcription. In addition, *MYB114*-like genes, *WRKY71* and *WRKY31* genes were identified in present study, which transcript level were up-regulated in ABA8, Suc8 and AS8 treatments, especially in AS8 treatment. *MYB114* has been reported to be associated with anthocyanin biosynthesis in *Arabidopsis* and pear (Gonzalez *et al.* 2008; Yao *et al.* 2017). The expression level of *TaWRKY71* was 3-folds higher in ABA treated-wheat than that of the control (Xu *et al.* 2014b). Therefore, our results indicated that *MYB114*-like, *WRKY71* and *WRKY31* were responsive to ABA and/or sucrose, and then further involved in activating anthocyanin biosynthesis.

Based on the structural genes of anthocyanin biosynthesis regulated by MBW complexes, the 'late' flavonoid biosynthetic steps depend on these complexes and 'early' steps that are not (Martin *et al.* 1991; Shirley *et al.* 1995; Pelletier and Shirley 1996; Pelletier *et al.* 1997; Zhang *et al.* 2003). *DFR*, *ANS* and *UFGT* genes play critical roles in anthocyanin biosynthesis (Zhao *et al.* 2012; Li *et al.* 2013; Gao *et al.* 2019). In our study, the transcripts level of *DFR* (Cluster-12337.70165), *UFGT* (Cluster-12337.64199) and *ANS* (Cluster-12337.69226 and cluster-12337.70413) were significantly up-regulated in ABA8, Suc8 and AS8 treatments, especially AS8 treatment. The transcripts level of *UFGT* (Cluster-12337.80836) was significantly up-regulated in Suc8 and AS8 treatments, and the transcripts level of *UFGT* (Cluster-12337.61103) was down-regulated in ABA8 and Suc8 treatments. Ban *et al.* (2003) reported that ABA treatment increased the anthocyanin content in grape skin with the upregulation of *DFR* and *UFGT* genes. In red-leaf peach, 0.3% sucrose could increase the expression level of *UFGT* (Wen *et al.* 2016). ABA/or sucrose treatments enhanced anthocyanin accumulation in increasing expression level of *VvUFGT* in grapes (Olivares *et al.* 2017). Our results suggested that these genes might be important structural genes involved in rapid accumulation of anthocyanins induced by ABA and sucrose in strawberry fruit. Glutathione S-transferase (GST) was commonly considered to be the detoxification of endogenous and xeno-biotic compounds in plants (Marrs 1996) and its role in response to biotic and abiotic stress has been clarified in *Arabidopsis* (Wagner *et al.* 2002). Overexpression of *ThGSTZ1* enhanced the salt and drought tolerance in *Tamarix hispida* (Gao *et al.* 2016). Meanwhile, Shi *et al.* (2014) and Kitamura *et al.* (2012) reported that GST protein was a positive regulator in the accumulation (Kitamura *et al.* 2012) and transport (Shi *et al.* 2014) of anthocyanins in *cyclamen* and *Paeonia delavayi*, respectively. The highest transcripts level of *GST* was detected as the anthocyanin content reached maximum in chili pepper (Aza-Gonzalez *et al.* 2013). Furthermore, ABA could induce the expression of *LcGST* in litchi (Hu *et al.* 2016) and *GmGST26A* in maize (Alfenito *et al.* 1997). In this study, the transcripts of 6 *GSTs* were up-regulated by ABA8, Suc8 and AS8 treatments, especially Suc8 treatment. This suggested that *GST* gene was involved in ABA and sucrose induced anthocyanin accumulation.

Auxin could inhibit ABA accumulation and fruit ripening related genes expression (Jia *et al.* 2017). In raspberry, auxin decreased the content of anthocyanins by down-regulating the expression of *MYB10* and *ANS* (Moro *et al.* 2017). Similar results were reported in carrot (Ozeki and Komamine 1986). Auxin responsive protein (Aux/IAA) and auxin response factor (ARF) were involved in auxin signaling pathway (Wang *et al.* 2018). When there was no auxin, Aux / IAA and ARF formed dimer to prevent ARF from binding with auxin response element (ARE), and then inhibited the transcription of auxin response genes. In the presence of auxin, Aux/IAA was degraded by ubiquitin, and then ARF and ARE bonded to each other, which led to the transcription of auxin responsive genes (Leyser 2002). In apple, auxin treatment upregulated the transcripts of 9 *Aux/IAAs* and 7 *ARFs* while the transcripts

of some *MYB* and *bHLH* and anthocyanins content were down-regulated (Ji *et al.* 2015). Overexpressed *MdARF5* decreased anthocyanin content by direct inhibition of *MdMYB1* expression and indirect inhibition of anthocyanin synthesis gene expression, respectively (An *et al.* 2018). In this study, the expression of *ARF5*, *Aux*/*IAA4* and *Aux*/*IAA16* was inhibited in ABA8, Suc8 and AS8 treatments, especially in AS8 treatment, suggesting that ABA and sucrose partially suppressed auxin signaling pathway to weaken the inhibitory effect of auxin on anthocyanin biosynthesis.

Conclusion

ABA, sucrose and ABA + sucrose treatments promoted anthocyanins accumulation and ABA + sucrose treatment had the greatest effect, which supplied the details of the effects of ABA and/or sucrose on the global transcriptome modification during strawberry coloration. Identification and expression analysis of MBW complex, *WRKY*, *Aux/IAA* and *ARF* showed that most of them may act as negative regulatory factors for anthocyanin synthesis. Strong transcript signals of *DFR*, *ANS*, *ANR* and *UFGT* in structural genes contributed to the accumulation of anthocyanins in sucrose and ABA + Sucrose treatment. Meanwhile, GST protein is also a positive regulator for anthocyanin accumulation.

Acknowledgements

The authors acknowledge the support from National Natural Science Foundation of China (3180817), Key projects of Sichuan Provincial Science and Technology Department (2018NZ0126) and the State Education Ministry, Key projects of Sichuan Provincial Education Department (172A0319) and Service Station Projects of New Rural Development Research Institute, Sichuan Agricultural University (2018, 2020).

Author Contributions

YL and XZ designed the experiments and wrote the manuscript. CG performed most of the experiments and analyzed results. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript.

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