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Abstract: Climate change is known to simultaneously increase berry sugars but decrease anthocyanins, leading to an imbalance between sugars and anthocyanins in grape berries. To restore the balance of sugars and anthocyanins, carbon limitation by leaf removal and exogenous abscisic acid (ABA) were separately or simultaneously applied to *Vitis vinifera* cv. Cabernet Sauvignon fruit cuttings to decipher their effects on berry quality with metabolite and whole-genome transcriptome analyses. Carbon limitation decreased the hexose concentration and fully blocked the accumulation of anthocyanins. However, exogenous ABA increased the anthocyanin concentration under both carbon limitation and sufficient conditions. Carbon limitation and exogenous ABA induced the profound remodeling of the whole-genome transcriptome and altered the anthocyanin concentration by regulating the transcription levels of genes involved in the anthocyanin biosynthesis pathways as well as in the genes involved in various types of hormone signaling. Moreover, two pertinent candidate genes were identified based on the co-expression network analysis between the berry metabolite and transcriptome results, including a transcriptional factor, *ERF2*, and a calcineurin B-like protein-interacting protein kinase gene, *CIPK25*. In summary, simultaneously modifying the carbon supply by leaf removal and spraying exogenous ABA could re-establish the balance between sugars and anthocyanins to improve the qualities of grape berries via whole-genome transcriptome remodeling.

Keywords: carbon limitation; ABA; decoupling; hexose; anthocyanin; transcription levels

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1. Introduction

The quality of grape berries is directly determined by the berries' chemical components, and climate change is a key factor influencing fruit components [1,2]. With the intensification of global warming, the concentrations of soluble sugar are increasing in grape berries, leading to an increase in the alcohol content in grape wine [3]. At the same time, high temperatures inhibit the synthesis of anthocyanin and promote its degradation, resulting in a decrease in the concentration of anthocyanin in berries [2,4,5], leading to an imbalance between sugars and anthocyanins and threatening the sustainable development of the wine industry.

Modifying the source–sink ratio is a common manipulation that influences the sugar content in berries [6–9]. Different carbon supplies influence the central carbohydrate metabolites in primary metabolism pathways [10,11]. A low source–sink ratio decreases the soluble sugar (sucrose, glucose, and fructose) in grape [12], orange [13], kiwifruit [14], and tomato [8,15]. In addition, carbon supply also alters secondary metabolites (e.g., anthocyanins, flavonols, and volatile organic compounds, etc.) [16–19]. The most concerning issue is

the responses of the anthocyanin concentrations in grapevine berries to carbon limitation. Carbon limitation significantly reduces the concentrations of anthocyanins in grape berries [12,20–25], and its effect on di-hydroxylated anthocyanins is more evident than that of tri-hydroxylated anthocyanins [23,26]. For example, in a semi-controlled greenhouse with the same environmental conditions as this study, carbon limitation reduced the sugar content by 27%, but the anthocyanin content was reduced by 84% in grape berries under the same carbon limitation level with Cabernet Sauvignon fruit cuttings [6]. From these results, it can be postulated that when the carbon supply is deficient, its effect on secondary metabolism is more pronounced than it is on primary metabolism, which finally leads to the uncoupling of sugar and anthocyanins. To re-establish the balance between sugars and anthocyanins in grape berries, the effects of combined carbon limitation and exogenous ABA application have been explored [23,24] at different metabolite levels in combination with gene expression analysis using the qPCR method.

ABA is a key hormone in regulating ripening in non-climacteric fruit [27,28]. The exogenous spraying of ABA on grape berries promoted the accumulation of sugars and anthocyanins, while ABA showed little influence on organic acids [23,24,29–31]. Moreover, Wang et al. [23,24] showed that exogenous ABA restored, at least partially, the imbalance between sugar and anthocyanins under different carbon supplies. To explore the transcriptional responses of target genes to exogenous ABA treatment and carbon supply, many studies have analyzed target gene expression by real-time quantitative PCR under the conditions of spraying ABA and different carbon supplies. The expression of anthocyanin's structural genes (*CHS*, *CHI*, *F3H*, *LDOX*, and *UFGT*) and regulator genes (*MYBA1* and *MYBA2*) were decreased under a low carbon supply, while the expression of those genes was increased by ABA treatment independent of carbon supply [24]. However, the molecular mechanism by which carbon supply and ABA regulate anthocyanin synthesis is still unclear. Previous gene expression analyses with qPCR were often limited by their throughputs, and a whole-genome transcriptome analysis will provide novel insights into transcriptome remodeling in response to carbon limitation and exogenous ABA.

In this study, we generated two different carbon supplies by modifying the leaf-to-fruit ratio and sprayed exogenous ABA exclusively or in combination with carbon supply treatments on berries from *Vitis vinifera* cv. Cabernet Sauvignon fruit cuttings. Comprehensively analyzing the quality traits associated with the transcription of berries during different developmental stages, we aimed to analyze the molecular mechanism of carbon supply and ABA influencing fruit quality and to identify pertinent candidate genes that regulate anthocyanin biosynthesis worth further molecular functional analysis.

2. Materials and Methods

2.1. Plant Materials, Treatments, and Sampling

The 1-year-old grapevine fruit cuttings (*Vitis vinifera* cv. *Cabernet Sauvignon*) were prepared in a naturally lit and semi-controlled greenhouse (the day temperature was controlled at about 26 °C, and the night temperature was controlled at about 20 °C) in Beijing, China, in 2021, as described in Mullins and Rajasekaran [32]. Every fruit cutting was automatically irrigated by a drip irrigation system with full-strength Hoagland's solution [33] three to seven times per day to avoid any water stress. Two levels of carbon supply and exogenous ABA spray treatments were applied at one week before veraison (59 days after flowering, 59 DAF). The two carbon supply levels included carbon-sufficient (CK) conditions, with 12 leaves per cluster, and carbon limitation (CL), with two leaves per cluster. In addition, exogenously sprayed ABA treatments (400 mg/L ABA) were conducted on the CK and CL treatments and named CK_ABA and CL_ABA, respectively. The ABA solution was evenly and exclusively sprayed on the berries at the beginning of the experiment and 7 days after the first treatment. During ABA spraying, the leaves of the fruit cuttings were protected with a plastic film to make sure that the ABA only reached the berries and not the leaves or any other organs. The remaining plants were sprayed with water [23,24]. In total, there were 4 treatments: CK, CL, CK_ABA, and CL_ABA.

These treatments were distributed in a randomized complete block design with 3 blocks for 3 biological replicates, and each biological replicate contained 5 fruit cuttings. Before the treatments, fruit cuttings with similar growth performance, such as similar leaf sizes and cluster sizes, were chosen.

Berries were sampled at 59, 69, 79, 89, 113, and 128 DAF. For each sampling date, 4 berries were sampled from different positions on each bunch, and finally, 20 berries taken from 5 clusters were pooled as a biological replicate, with a total of 3 biological replicates for each treatment. Samples were rapidly frozen in liquid and stored at −80 °C. After removing pedicels, berries were weighted to determine their fresh weight. The seeds were removed from the berries, and the skin and pulp were ground into fine powders in liquid nitrogen with a ball grinder: N9548 (HODER, Beijing, China).

2.2. Extraction and Determination of Soluble Sugars

An aliquot of 200 mg berry fresh powder was freeze-dried for 48 h; then, the dried powder was extracted with 1 mL deionized water at 80 °C for 15 min and centrifuged (5000 rpm for 10 min), and the supernatant was transferred to another tube. Then, the precipitate was extracted twice with 1 mL deionized water following the same procedure. The supernatant was mixed and filtered through a 0.22 μM filter [12]. The concentrations of soluble sugar (glucose and fructose) were measured with a Waters 2695 high-performance liquid chromatography (HPLC) system with a Waters RI-2414 detector (Waters, Milford, MA, USA) according to Kuang et al. [34] and Zhang et al. [35].

2.3. Extraction and Determination of Anthocyanins

An aliquot of 500 mg berry fresh powder was freeze-dried for 48 h, and then the dried powder was extracted in 1 mL 2% formic acid–methanol solution (*v*/*v*) [36]. The extracts were used to analyze anthocyanins after filtering through a 0.22 μM nylon filter. Each individual anthocyanin was measured using an UltiMate 3000 HPLC system with an UltiMate 3000 DAD detector (Thermo Fisher Scientific, Waltham, MA, USA) at 520 nm, as detailed in Dai et al. [37] and Hilbert et al. [38]. The separation column was a reversed-phase Inertsil^{TM} ODS-3 column (25 cm × 4.6 mm, 5 μm particle size, GL Sciences Inc., Tokyo, Japan) at 25 °C with elution at 0.6 mL/min according to a binary gradient: 0 min 80% A 20% B, 70 min 15% A 85% B, 75 min 80% A 20% B, and 80 min 80% A 20% B (solvent A: water and formic acid, 90/10 *v*/*v*; solvent B: acetonitrile and formic acid, 30/10 *v*/*v*). The integration was executed with Chromeleon software v.7.1 (Thermo Fisher Scientific, Waltham, MA, USA), and malvidin-3-*O*-glucoside (Extrasynthese, Lyon, France) was used as the standard for all of the anthocyanins.

2.4. Transcriptome Sequencing and RNA Sequencing Data Analysis

The berries sampled at 69, 79, and 89 DAF were analyzed for RNA sequencing (RNA-seq). Total RNA of the samples was extracted with a Biomarker plant total RNA isolation kit (Biomarker, Beijing, China). The quantifications of the total RNA were examined by a NanoDrop 2000 and an Agilent 2100 Bioanalyzer. Then, the cDNA libraries were constructed using a VAHTS mRNA-seq V3 Library Prep Kit for Illumina® (Vazyme, Nanjing, China), and mRNA was sequenced on an Illumina Novaseq 6000 by Biomarker Technologies (Beijing, China).

The clean data were obtained though removing low-quality sequences and adapters from the raw count data using Trimmomatic software v.0.39 [39]. Then, the obtained high-quality sequences were mapped to the grape reference genome PN40024 12X. v2.1 by STAR (v.2.7.9a, https://github.com/alexdobin/STAR, accessed on 5 December 2021) [40]. The expression levels were calculated by RSEM (v.1.3.1, https://github.com/deweylab/RSEM, accessed on 5 December 2021) and were normalized into FPKM (fragments per kilobase of transcript per million mapped reads) values. The DEGs (differentially expressed genes) were filtered based on the adjusted *p*-values ($p < 0.05$) and fold changes (fc > 2) by DESeq2 R package (v.1.34.0, http://www.bioconductor.org/packages/release/bioc/

html/DESeq2.html, accessed on 5 December 2021) [41]. Gene annotation was performed with AnnotationHub R package (v.3.2.0, https://bioconductor.org/packages/release/bioc/html/AnnotationHub.html, accessed on 5 December 2021).

The common up- and downregulated genes among the different developmental stages were identified with TBtools (v.1.098685, https://github.com/CJ-Chen/TBtools, accessed on 5 December 2021) [42]. Gene ontology (GO) enrichment of common genes was analyzed with clusterProfiler (v.4.2.1, https://github.com/YuLab-SMU/clusterProfiler, accessed on 5 December 2021) [43].

2.5. Network Analysis and Visualization

The network was constructed according to the pairwise significant Pearson correlations between the anthocyanin concentrations and DEGs with a *p*-value ($p < 0.000001$). The network was visualized with Cytoscape (v.3.9.0, https://cytoscape.org/, accessed on 5 December 2021) [44].

2.6. Statistical Analysis

The data analysis and graphing were conducted in R software v.4.1.1 [45]. Significance between the treatments was analyzed by ANOVA and Tukey's post hoc test ($p < 0.05$).

3. Results

3.1. The Effect of Carbon Limitation and Spraying ABA Treatment on Grape Berry Metabolites

The berry fresh weight, relative water content, hexose (glucose and fructose) concentration, and berry coloration showed different responses to carbon limitation and exogenous spraying with ABA treatment (Figure 1). Carbon limitation significantly decreased the berry weight compared to the carbon-sufficient group (Figure 1A). However, exogenous ABA had no significant effect on berry fresh weight compared to the treatments without ABA (Figure 1A). Water content was significantly increased by carbon limitation compared to the carbon-sufficient group, while water content was reduced by the exogenous ABA treatments compared to the groups without ABA treatment (Figure 1B). Furthermore, carbon limitation resulted in an average decrease of 25.2% in the hexose concentration compared to the carbon-sufficient group, while exogenous ABA led to no significant influence on the hexose concentration compared to the treatments without ABA (Figure 1C).

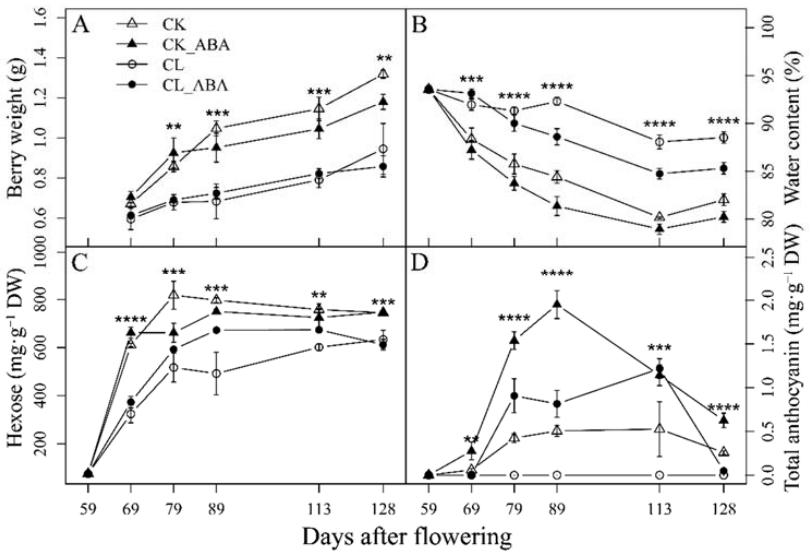

Figure 1. The effect of carbon limitation and exogenous ABA treatment on the berry fresh weight (**A**), water content (**B**), hexose concentration (**C**), and the total anthocyanins (**D**) during different developmental stages. CK: carbon sufficient; CL: carbon limitation; CK_ABA: carbon-sufficient and exogenous ABA treatment; CL_ABA: carbon limitation and exogenous ABA treatment. Vertical bars indicate SE ($n = 3$). ** indicates $p < 0.01$; *** indicates $p < 0.0001$; and **** indicates $p < 0.00001$.

Changes in berry coloration were completely inhibited under carbon limitation over the course of berry development, while the veraison of berries under carbon-sufficient conditions occurred at 71 DAF, and almost all of the berries changed color at 86 DAF (Figure S1). The change in berry coloration was much faster under the exogenous ABA treatment than when there was no ABA treatment (Figure S1A). The beginning of color change in the CK_ABA berries was at 7 days after treatment (66 DAF). The berries under CK_ABA changed color at a rapid rate (10% per day) and were fully colored at 72 DAF (Figure S1B). The beginning of coloring in the CK_ABA berries was 5 days earlier than the CK berries. The exogenous ABA also promoted the CL berries to color (Figure S1A). The CL_ABA berries began to change color at 69 DAF, and the rate of color change (5.8% per day) was faster than that of CK berries (3.3% per day) (Figure S1B). In addition, carbon limitation significantly decreased the total anthocyanin concentration compared to carbon-sufficient conditions. Compared to no ABA treatment, the total anthocyanin concentration was increased with exogenous ABA treatment, except at harvest (128 DAF), when the total anthocyanin concentration of CL_ABA berries was lower than that of CK berries due to the rapid degradation of anthocyanin (Figure 1D).

3.2. mRNA Quantities of Genes Related to Anthocyanin Biosynthesis and Hormone Signaling Pathways

To investigate the mRNA quantity responses of berries to carbon limitation and exogenous ABA treatment, we conducted RNA-seq for 36 cDNA libraries for four treatments with three stages (69 DAF, 79 DAF, and 89 DAF) and three biological replicates. A total of 864.09 million clean reads were obtained from 36 cDNA libraries, and the average mapping rate was 89.59%. Principal component analysis (PCA) plots revealed clear separation among different treatments (Figure S2), indicating that the RNA-seq data were reliable for further analysis.

There were 22 genes related to anthocyanin biosynthesis, with significantly different expression occurring in one of the four treatments or at three developmental stages ($|\log_2 FC| > 1$ and FDR ≤ 0.05, Figure 2). Most of the structural and regulator genes related to anthocyanin biosynthesis were significantly different among different treatments. The mRNA quantities of structural genes (*PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *F3′5′H*, *DFR*, *LDOX*, *UFGT*, *MATE*, *ABCC,* and *GST*) were downregulated under carbon limitation, but the mRNA level of 4CL was not significantly influenced under carbon limitation compared to under carbon-sufficient conditions. The mRNA levels of the regulator genes (*MYBA2* and *MYBPA1*) were also downregulated under carbon limitation, while the mRNA levels of the negative regulators (*MYB4B* and *MYBC2-L3*) were upregulated under carbon limitation. However, the mRNA quantity of *MYBA1* was not significantly affected under carbon limitation compared to carbon-sufficient conditions.

Exogenous ABA treatment increased the mRNA quantities of all of the structural genes related to anthocyanin synthesis under both carbon-sufficient conditions and carbon limitation as well as the positive regulators (*MYBA1*, *MYBA2,* and *MYBPA1*). However, the carbon-sufficient and ABA treatment (CK_ABA) had no significant effect on the expression of the *MYB4B* and *MYBC2-L3* genes compared to the carbon-sufficient condition alone, while carbon limitation and ABA treatment (CL_ABA) significantly reduced the expression of *MYB4B* and *MYBC2-L3* compared to carbon limitation alone.

In addition, the expression of flavonol synthesis genes (*FLS*), which are involved in phenylpropanoid pathways, was decreased under carbon limitation. Exogenous ABA showed little effect on the expression of *FLS*. The tannin synthesis gene (*LAR*) did not respond to carbon limitation, while ABA increased the mRNA levels of *LAR* only at 69 DAF.

To explore the effects of carbon supply and ABA treatment on different hormone signaling processes in berries, we selected significant different genes related to ABA, auxin, brassinosteriod, cytokinine, ethylene, gibberellin, jasmonic acid, and salicylic acid synthesis and signaling and analyzed the expression of these genes under carbon supply and ABA

treatment (Figure 3). ABA treatment (CK_ABA vs. CK, CL_ABA vs. CL) increased the transcript levels of the ABA synthesis (*VvNCED*) and ABA signaling pathway (*VvPP2C*, *VvPYR/PYL*, and *VvSnRK2*) genes. The transcript levels of genes related to cytokinine signaling (*VvCRE1*), ethylene biosynthesis (*VvACO* and *VvCTR1*), jasmonic acid signaling (*VvJAZ*), and salicylinc acid signaling (*VvTGA*) were increased under ABA treatment compared to no ABA treatment (CK_ABA vs. CK, CL_ABA vs. CL). The transcript levels of genes related to auxin signaling (*VvARF*, *VvAUX/IAA*, and *VvSAUR*) and gibberellin signaling (*VvDELLA*, *VvGID1*, and *VvPIF3*) were downregulated under ABA treatment (CK_ABA vs. CK, CL_ABA vs. CL). Exogenous ABA treatment showed little effect on the mRNA quantity of genes related to brassinosteriod signaling (*VvBRII*).

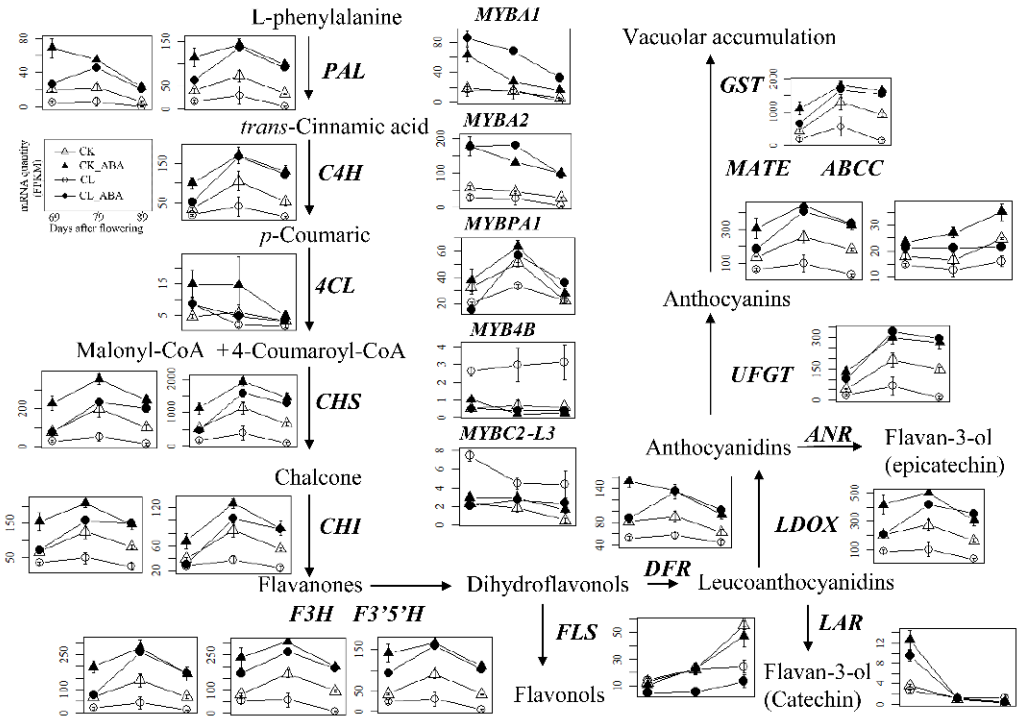

Figure 2. The mRNA quantities of genes related to anthocyanin biosynthesis at the first three developmental stages under different carbon supplies and exogenous ABA treatments. *PAL*: pheammonia-lyase; *C4H*: cinnamate 4-hydroxylase; *4CL*: 4-coumarate-CoA ligase; *CHS*: chalcone synthase; *CHI*: chalcone isomerase; *F3H*: flavanone 3-hydroxylase; *FLS*: flavonol synthase; *DFR*: dihydroflavonol 4-reductase; *LAR*: leucoanthocyantin reductase; *LDOX*: leucoanthocyanidin dioxygenase. *ANR*: anthocyanin reductase; *UFGT*: UDPG-flavonoid-3-*O*-glucosyltransferase; *GST*: giutathione S-transferase; *MATE*: multidrug toxic compound extrusion; and *ABCC*: ATP binding cassette.

With no ABA treatment, all of the hormone biosynthesis and signaling genes were slightly affected by carbon limitation (CL vs. CK). For example, although the transcript level of *VvNCED* was upregulated by carbon limitation, $\log_2$fc was lower than 1.5. With ABA treatment, carbon limitation (CL_ABA vs. CK_ABA) downregulated one of the ABA signaling genes (*VvPP2C*, VIT_216s0050g02680), while carbon limitation showed little effect on the mRNA levels of other ABA signaling genes. In addition, carbon limitation increased the transcript levels of auxin signaling genes (*VvARF*, VIT_215s0046g00290; *VvAUX/IAA*, VIT_211s0016g03540; and *VvSAUR*, VIT_209s0002g00670) and gibberellin signaling genes (*VvDELLA* and *VvGID1*), while carbon limitation downregulated the mRNA quantities of ethylene signaling genes (*VvACO*) via the exogenous spraying of ABA.

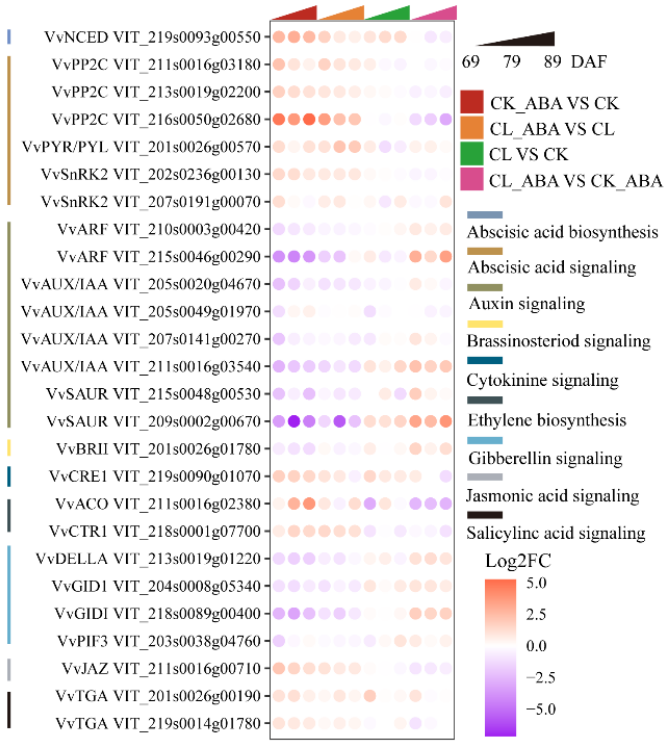

Figure 3. The effects of carbon limitation and exogenous ABA treatment on the mRNA levels of hormone biosynthesis and signaling genes during the first three developmental stages. CK_ABA vs. CK indicate the differential changes in genes in the CK_ABA treatment vs. CK as control. The following three combinations have the same meaning as above. The three columns from left to right corresponding to each combination represent the changes in gene expressions at 69 DAF, 79 DAF, and 89 DAF. In the dot plot, red represents upregulation of mRNA levels in genes, and purple represents downregulation of mRNA levels in genes. The colors of circles represent the intensity of the fold changes in gene expressions (log_2FC), and the white represents no influence on the gene expression.

3.3. Transcriptomic Response of Berries to Exogenous ABA Treatment under Different Carbon Supplies

To assess the transcriptomic responses of berries to exogenous ABA treatment under different carbon supplies, we analyzed the number and function of differential expression genes (DEGs) under ABA treatment with carbon limitation and under carbon-sufficient conditions. Compared to carbon limitation, ABA treatment (CL_ABA vs. CL) led to 363 DEGs being commonly upregulated (Figure S3A) and 421 DEGs being commonly downregulated for the first three developmental stages (Figure S3B). As both the berries treated under carbon-sufficient (CK) and carbon-sufficient with exogenous ABA (CK_ABA) conditions were completely colored at 69 DAF, we determined the common DEGs between 69 DAF and 79 DAF in order for some DEGs related to anthocyanin to not be excluded. Therefore, compared to carbon-sufficient conditions, ABA treatment (CK_ABA vs. CK) led to 100 DEGs being commonly upregulated (Figure S3C) and 72 DEGs being commonly downregulated for the first two developmental stages (Figure S3D).

Then, we conducted the overlap of these common DEGs (Figure 4A). There were 363 DEGs that were only downregulated in the comparison of CL_ABA vs. CL, named Z363, and 286 DEGs that were only upregulated in the comparison of CL_ABA vs. CL, named Z286. In total, 77 DEGs were upregulated in both the comparisons of CK_ABA vs. CK and CL_ABA vs. CL (Z77), while 58 DEGs were downregulated in both of the comparisons (Z58). There were 23 upregulated DEGs (Z23) and 14 downregulated DEGs (Z14) that only occurred in the comparison of CK_ABA vs. CK.

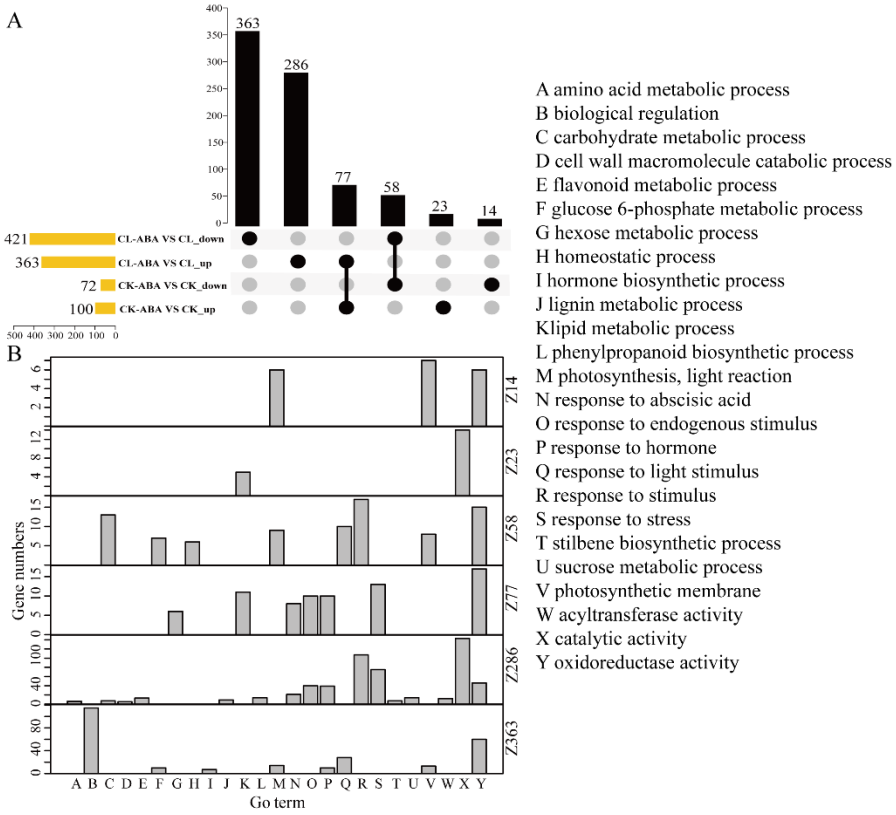

Figure 4. The number and functional enrichment of genes in different combinations under ABA treatment with different carbon supplies. (**A**) The number of DEGs between different combinations. The overlap of DEGs in different combinations were visualized with UpSetR. The vertical bars represent the number of DEGs within each intersect, while the filled circles below show the combinations for established intersect. Two connected circles indicate that these DEGs are present in both combinations. The horizontal bars represent the total number of DEGs for each combination. (**B**) The number of the selection of significant enrichment terms from GO enrichment analysis for the DEGs in different zones. The six different combinations are named Z (zones), with the DEG counts of different zones found on the right-axis labels in (**B**), and the different GO terms are represented by capital letters.

GO enrichment analysis of each zone (Figure 4B) showed that the DEGs upregulated by spraying ABA during carbon limitation and on carbon-sufficient berries (Z77) were enriched in their hexose and lipid metabolic processes (G and K) as well as in their responses to abscisic acid, endogenous stimuli, hormones, and stress (N, O, P, and S). The DEGs that were only upregulated by spraying ABA during carbon limitation (Z286) were also enriched in their responses to hormones and stress (N, O, P, R, and S), while the GO terms enriched by the DEGs that were only upregulated by spraying ABA in carbon-sufficient conditions (Z23) were not related to the response to hormones. In both the carbon limitation and carbon-sufficient conditions, the DEGs downregulated by spraying ABA (Z363, Z58, and Z14) were enriched in photosynthesis and in the photosynthesis membrane (M and V). In addition, the DEGs that were only downregulated by spraying ABA during carbon limitation (Z363) were also enriched in the glucose 6-phosphate metabolic process, hormone biosynthetic process, response to hormones, and response to light stimulus (F, I, P, and Q). However, the DEGs that were only downregulated by spraying ABA under carbon-sufficient conditions (Z14) were not related to these functions.

3.4. Transcriptomic Response of Berries to Carbon Limitation under ABA or Non-ABA Treatment

We analyzed the number and function of DEGs under carbon limitation receiving ABA and non-ABA treatment (Figure S4). Under non-ABA treatment and compared to carbon-sufficient conditions, carbon limitation (CL vs. CK) led to 485 commonly upregulated DEGs

(Figure S4A) and 126 commonly downregulated DEGs (Figure S4B) among the first three developmental stages. Under ABA treatment, carbon limitation (CL_ABA vs. CK_ABA) led to 183 commonly upregulated DEGs (Figure S4C) and 120 commonly downregulated DEGs (Figure S4D) in the first two developmental stages.

Then, we conducted the overlap of these common DEGs (Figure 5A). There were 382 upregulated DEGs (Z386) and 93 downregulated DEGs (Z93) in the comparison of CL vs. CK. There were 80 upregulated DEGs (Z80) and 87 downregulated DEGs (Z87) in the comparison of CL_ABA vs. CK_ABA. There were 103 upregulated DEGs (Z103) and 33 downregulated DEGs (Z33) when comparing both CL vs. CK and CL_ABA vs. CK_ABA.

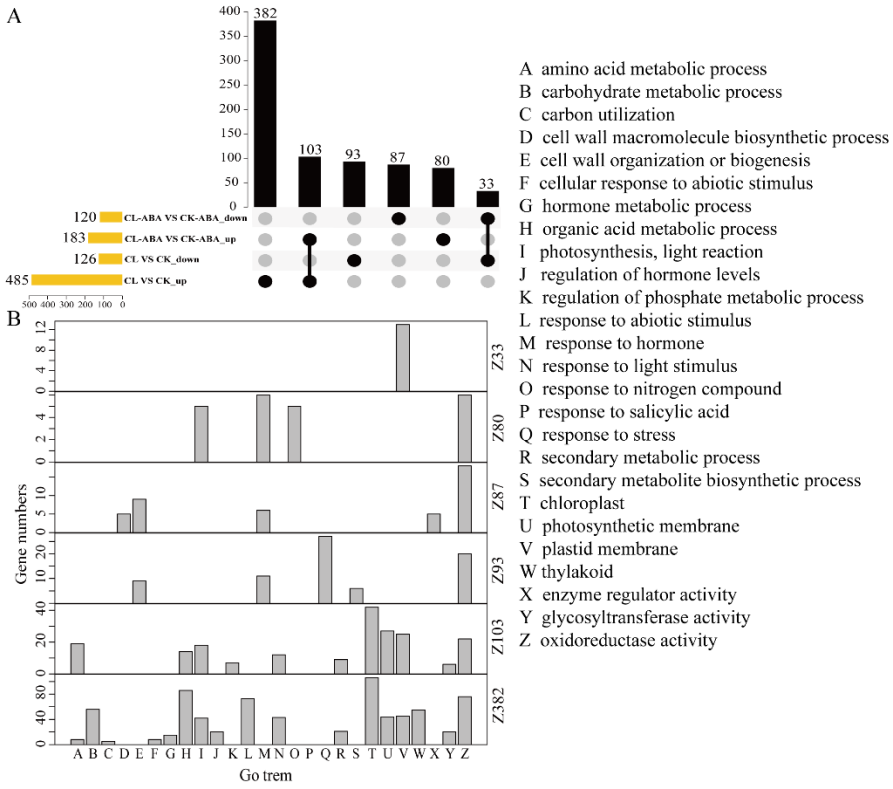

Figure 5. The number and functional enrichment of genes in different zones under carbon limitation receiving ABA or non-ABA treatment. (**A**) The number of DEGs between different combinations. (**B**) The number of significantly enriched terms from GO enrichment analysis for the DEGs in different zones. The organization of the figure is the same as detailed in Figure 4.

GO enrichment analysis of each zone (Figure 5B) showed that the DEGs upregulated under carbon limitation receiving ABA and non-ABA treatment (Z103) were enriched in the amino acid and organic acid metabolic processes (A and H), secondary metabolic processes (R), and in photosynthesis and the organelles associated with photosynthesis (I, T, U, and V). Although the DEGs that were upregulated in CL berries (Z382) were also enriched in the same terms (A, H, R, I, T, U, and V), the DEGs that were only upregulated in CL_ABA berries (Z80) were enriched in photosynthesis and in their response to hormones (I and M). The DEGs downregulated under carbon limitation with non-ABA treatment (Z93) were enriched in their responses to hormones and stress and in secondary metabolite biosynthesis processes (M, Q, and S). The DEGs downregulated under carbon limitation with ABA treatment (Z80) were only enriched in response to hormone response (M). However, the DEGs downregulated under carbon limitation with ABA and non-ABA treatment (Z33) were not related to these functions (M, Q, and S).

3.5. The Correlation Network of Genes Related to Anothocynain

In order to screen some of the target genes related to anthocyanin accumulation, firstly, we analyzed the correlations among the data for the anthocyanin concentration and the mRNA levels of 5337 DEGs from the berries under the four treatments in the first three developmental stages. In total, 288 DEGs were significantly correlated with anthocyanin concentrations ($p < 0.000001$), including known structural genes and transporter proteins related to anthocyanin, sugar metabolism genes, transcription factors, and some genes of unknown function. Then, we took the 18 known anthocyanin genes as targets and performed correlations with the other 270 genes. Since the correlations between genes was extremely high, we screened them with an absolute value of the correlation coefficient ($|cc| > 0.85$), and a total of 160 genes were significantly correlated with the target genes. Based on the gene annotations, 14 known structural genes and 15 transcription factors were selected and mapped into a correlation network (Figure 6).

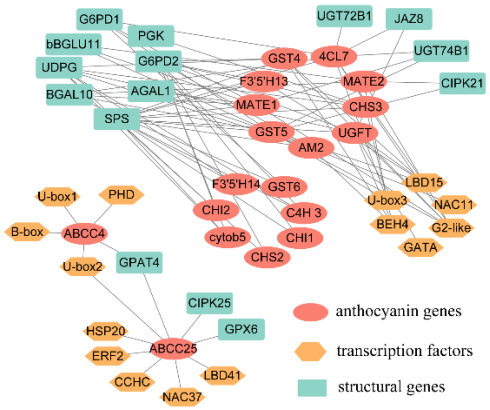

Figure 6. Integrated co-expression network of anthocyanin-related genes.

The transcription factors in the network included two *NAC* transcription factors (*NAC11* and *NAC37*); two LOB domain–containing transcription factors (*LBD15* and *LBD41*); an ethylene response factor (*ERF2*); zinc finger (*CHY-type*, *B-box*, *CCHC*, *PHD*, and *GATA*) and protein phosphatase 2C (*PP2C*); *G2-like*; three U-box transcription factors (*U-box1-3*) and a heat shock protein (*HSP20*); and a BES1/BZR1 homolog transcription factor in the brassinosteroid signaling pathway (*BEH4*). Moreover, 14 structural genes were also significantly correlated with anthocyanin genes in the network, including two carbon metabolism genes encoding the glucose 6-phosphate dehydrogenase (*G6PD1* and *G6PD2*), a gene encoding the phosphoglycerate kinase (*PGK*), a β-galactosidase gene *BGAL10*, an *α*-galactosidase gene (*AGAL1)*, a β-glucosidase gene (*bBGLU11)*, the uridine diphosphate glucosyltransferase gene *UDPG*, and a sucrose phosphate synthase gene (*SPS)*. The other structural genes included the JASMONATE ZIM domain protein (*JAZ8*) as a repressor of the jasmonic acid pathway, the glycosyltransferase genes *UGT74B1* and *UGT72B1*, the calcium-regulated phosphatase B-like protein intercalating protein kinases *CIPK21* and *CIPK25*, the glutathione peroxidase gene *GPX6*, and the glycerol 3-phosphate acyltransferase gene *GPAT4*.

4. Discussion

Ongoing climate change is affecting berry quality, and several strategies have been explored to mitigate its effects, including innovating vinicultural techniques [23,24] and breeding more resilient cultivars [46]. Consistent with previous studies, the concentrations of hexose and total anthocyanins decreased under carbon limitation [12,23,24,47]. However, the hexose concentration decreased by 36.8% under carbon limitation in berries, whereas the synthesis of anthocyanins was completely inhibited, with a 100% decrease. Therefore, we confirmed that anthocyanins were more sensitive to carbon supply than the hexose concentration, and this result led to the uncoupled hexose–anthocyanins under carbon

limitation. Because of the competition between primary and secondary metabolism [48], the only available carbon source would be preferentially allocated to primary carbohydrate metabolism to ensure the normal growth and development of berries. However, exogenous ABA effectively promoted the synthesis of anthocyanins in berries under carbon-sufficient conditions and carbon limitation. As shown in the results, the upregulated DEGs under carbon limitation (Z382) were enriched in carbohydrate metabolic processes, while the upregulated DEGs under carbon limitation with exogenous ABA (Z77 and Z286) were mostly enriched in the function of response to hormones, and most of the downregulated DEGs (Z58 and Z363) were related to carbohydrate metabolism. Therefore, more carbon seemed flow to the secondary metabolism pathway for anthocyanin synthesis under carbon limitation with exogenous ABA stimulus. In addition, the concentrations of the total anthocyanins under carbon limitation with exogenous ABA were higher than those in the CK berries, and this result was different from the result of Wang et al. [23], who showed that exogenous ABA under carbon limitation could only partially compensate for the anthocyanin decrease caused by carbon limitation. This difference might be attributed to the different methods of ABA treatment, as we sprayed ABA two times ABA: at beginning of the treatment and 7 days after the treatment, while Wang et al. [23] only treated with ABA once at the beginning of their experiment. It is possible that the secondary spraying resulted in a stronger stimulation effect on the accumulation of anthocyanins in the CL_ABA berries. This result also indicates that not only the concentration of ABA affects the accumulations of anthocyanins [27,29], but also that the frequency of spraying ABA affects the accumulation of anthocyanins in grape berries [30].

Exogenous ABA increased the mRNA levels of structural genes (*PAL*, *C3H*, *CHS*, *CHI*, *F3′5′H*, *DFR*, *LDOX*, and *UFGT*) and positive regulators (*MYBA1*, *MYBA2*, and *MYBPA1*) in the pathway of anthocyanin synthesis, while carbon limitation decreased the mRNA quantities of these structural genes and positive regulators. The mRNA levels of negative regulators (*MYB4B* and *MYBC2-L3*) [49] were increased under carbon limitation and were decreased with exogenous ABA under carbon limitation. These results suggest that ABA promotes anthocyanin synthesis by activating the expression of the genes involved in the pathway of anthocyanin synthesis [30,50,51]. To further investigate the mechanism by which exogenous ABA promotes anthocyanin accumulation, we detected the response of the transcription levels of ABA biosynthesis and ABA signaling genes to exogenous ABA. All of ABA signaling genes and *VvNCED*, a major structural gene in the ABA synthesis pathway, were activated with exogenous ABA, suggesting that the exogenous spraying of ABA also promotes endogenous ABA biosynthesis in berries, as previously found [23,24]. In addition, hormones synergistically regulated fruit development [52]. We found that the expression of genes related to cytokinin, ethylene, jasmonic acid, and salicylic acid signaling were activated when exogenous ABA was sprayed, suggesting that exogenous ABA also activates or inhibits the signaling functions of other endogenous hormones and that they may act together to promote the synthesis of fruit anthocyanins.

A total of 15 candidate transcription factors potentially related to anthocyanin synthesis were screened by the correlation network analysis between the anthocyanin levels and gene mRNA levels. Among them, the expression of an ethylene-responsive transcription factor, *ERF2* (VIT_216s0013g00890), was decreased under carbon limitation, while it was increased with exogenous ABA treatment (Figure S5A). The expression of *ERF2* was activated after veraison in berries (Figure S5B) [53]. Some *ERFs* in apple [54,55] and 'Red Zaosu' pear [56] regulate anthocyanin synthesis, but no *ERFs* have been reported to be related to anthocyanin synthesis in grapevine. *MdERF109* [54] and *MdERF38* [55] promoted the synthesis of anthocyanins by activating the promoters of downstream structural genes of anthocyanin. In 'Red Zaosu' pear, *Pp4ERF24* and *Pp12ERF96* interacted with *PpMYB114,* and the interaction enhanced the interaction between *PpMYB114* and *PpbHLH3*. Further analysis by a dual luciferase assay confirmed that both *ERFs* increased the expression of *PpUFGT* and promoted the synthesis of anthocyanin in 'Red Zaosu' pears [56]. Based on

the existing functional studies on *ERF* genes, we speculate that it is possible the *ERF2* in grape also has a role in promoting anthocyanin synthesis.

A calcineurin B-like protein-interacting protein kinase gene, *CIPK25* (VIT_204s0008g05770), was significantly correlated with the total anthocyanin concentration. The mRNA level of *CIPK25* was downregulated under carbon limitation while it was upregulated with exogenous ABA (Figure S6A). The expression of *CIPK25* was activated after veraison in berries (Figure S6B) [53]. *CIPKs* are a class of serine/threonine protein kinase that plays a key role in plant growth and response to stress. A total of 20 *CIPK* gene members were identified in grapevine genomes [57], and most of the *CIPK* genes respond to salt stress [58], drought stress [58,59], low temperatures [60], and ABA stimulus [61] in plants. However, no studies have reported that the functions of *CIPK* genes are related to anthocyanin synthesis. However, Yan et al. [62] found that the *AtCIPK14* gene can positively regulate the glucose signaling in *Arabidopsis*. Several studies have revealed that sugar also can act as signal molecule to regulate anthocyanin synthesis [63–66], so whether *CIPK25* can play a role in sugar signaling to regulate anthocyanin synthesis in grape berries deserves further exploration.

5. Conclusions

The decrease in anthocyanin concentration was more sensitive than the decrease in the hexose concentration under carbon limitation. Exogenous ABA effectively promoted anthocyanin biosynthesis independent of the carbon supply in berries. Hence, ABA can be used to increase the anthocyanin concentration when the hexose concentration is decreased by leaf removal to cope with the impact of climate change. Combined with the transcriptome, both carbon limitation and ABA affect anthocyanin synthesis by regulating anthocyanin-related genes (*PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *F3′5′H*, *DFR*, *LDOX*, *UFGT*, *MATE*, *ABCC*, *GST*, *MYBA1*, *MYBA2*, *MYBPA1*, *MYB4B*, and *MYBC2-L3).* Exogenous ABA can promote the synthesis of endogenous ABA and coordinate multiple hormones to promote anthocyanin synthesis. In addition, there are two candidate genes (*ERF2* and *CIPK25*) that may be related to anthocyanin synthesis, and we need to further verify their functions and mechanisms through molecular biology experiments. This will provide a solid theoretical basis for analyzing the mechanisms controlling the balance of sugar–anthocyanin in grape berries, with the aim of improving the quality of grapes and wine.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8100905/s1, Figure S1: The change of pigment and percentage of veraison of berries during different development stages under carbon limitation and exogenous ABA treatments. Figure S2: Principal component analysis of berries under different carbon supplies and exogenous ABA treatment during the first three development stages with transcriptome data (n=3). Different colors indicated the different treatments and different shapes indicated the different development stages. Figure S3: Venn diagram presenting the number of DEGs under ABA treatment with different carbon supplies. Figure S4: Venn diagram presenting the number of DEGs under carbon limitation with ABA or non-ABA treatment. Figure S5: The mRNA quantities of *ERF2* in fruiting cuttings (A) and in different organs during different development stages based on the online transcriptome data (B). Figure S6: The mRNA quantities of *CIPK25* in fruiting cuttings (A) and in different organs during different development stages based on the online transcriptome data (B).

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Abbreviations

CK	carbon sufficient
CL	carbon limitation
ABA	abscisic acid
CK_ABA	carbon sufficient and exogenous ABA treatment
CL_ABA	carbon limitation and exogenous ABA treatment

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