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Identification of Two *R2R3-MYB* Genes Involved in Flavan-3-Ols Biosynthesis as Modulated by Salicylic Acid Through RNA-Seq in Grape Berries (*Vitis* spp.)

Fengchan Yu [1], Pengfei Zhai [2], Wenjing Ge [1], Runtian Zhou [1], Shuwen Zan [1], Jinjun Liang [1], Pengfei Wen [1,3,*] and Yu Gary Gao [4,*]

[1] College of Horticulture, Shanxi Agricultural University, Jinzhong 030801, China; yufengchan2024@126.com (F.Y.); jingwg1005@163.com (W.G.); z1999rt@163.com (R.Z.); zsw15534249628@163.com (S.Z.); liangjinjun1989@163.com (J.L.)
[2] Department of Mechanical Engineering, Shanxi Institute of Technology, Yangquan 045000, China; zz-zpf@163.com
[3] Shanxi Key Laboratory of Germplasm Improvement and Utilization in Pomology, Shanxi Agricultural University, Jinzhong 030801, China
[4] South Centers, College of Food, Agricultural, and Environmental Sciences, The Ohio State University, 1864 Shyville Road, Piketon, OH 45661, USA
* Correspondence: sxauwpf@sxau.edu.cn (P.W.); gao.2@osu.edu (Y.G.G.); Tel.: +86-136-3354-8791 (P.W.)

Abstract: Flavan-3-ols are plant secondary metabolites that play important roles in stress resistance. Our previous studies revealed that salicylic acid (SA) activates *R2R3-MYB* transcription factors, promoting flavan-3-ol biosynthesis. This study identified two *R2R3-MYB* genes that exhibited positive responses to both exogenous SA and were probably involved in flavan-3-ol biosynthesis through RNA-sequencing, functional enrichment analysis, and qRT-PCR. The results indicated that the contents of total flavan-3-ols and their monomers, (+)-catechin and (−)-epicatechin, in grape berries after exogenous SA application were substantially increased compared to those in the control. A total of 683 differentially expressed genes in response to exogenous SA treatment were identified using RNA-seq. KEGG analysis revealed enrichment of the 'flavonoid biosynthesis' and 'plant hormone signal transduction' pathways. A specific module highly associated with flavan-3-ol biosynthesis was identified by constructing a co-expression network. Two candidate genes (*VvMYB108B* and *VvMYB145*) likely participating in flavan-3-ol biosynthesis were selected using qRT-PCR. Therefore, these two potential genes that respond to SA and putatively participate in flavan-3-ol biosynthesis were identified for the first time. These results lay a solid basis for a more profound understanding of the molecular regulation of flavan-3-ol biosynthesis in grapes.

Keywords: *Vitis vinifera*; salicylic acid; flavan-3-ols; RNA-seq; WGCNA

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1. Introduction

Grapevines (*Vitis vinifera* L.) are an economically valuable fruit crop worldwide [1] that produces a significant amount of proanthocyanidins (PAs) in its berries, leaves, and flowers. Grape-derived PAs influence mouthfeel by enhancing the astringency of fruits and in their processed products, e.g., wines and beverages [2]. Moreover, as potential dietary antioxidants, PAs are considered beneficial to human health [3]. PAs are polyphenolic mixtures bound by (+)-catechin and (−)-epicatechin. Several well-characterized enzymes, such as phenylalanine ammonia-lyase (PAL), dihydroflavonol 4-reductase (DFR), anthocyanidin reductase (ANR), and leucoanthocyanidin reductase (LAR), are crucial for flavan-3-ol accumulation in grapevines [4]. LAR and ANR may possess additional features that need to be explored [5].

These flavan-3-ol-specific structural genes are mainly regulated by the MBW complex, which includes *MYB*, *bHLH*, and *WD40*. In *Arabidopsis* seeds, TT2, TT8, and TTG1 are the

major transcription factors (TFs) that regulate PA biosynthesis [6]. AtMYB4 and AtMYB7 suppress phenylpropanoid biosynthesis [7]. AtMYB5 is linked to flavan-3-ol biosynthesis [8]. In strawberries, FaMYB5 and FaMYB10 facilitate PA accumulation through the LAR and ANR pathways [9]. In grapevines, VvMYBPA1, VvMYBPA2, and VvMYBPAR are known activators of PA metabolism mainly by targeting VvLAR1 and VvANR [10,11]. Recently, Cheng et al. demonstrated that VvMYB86 induces PA biosynthesis [12]. LAR and ANR are thought to be regulated by MBW ternary complexes. VabHLH137 enhances PA biosynthesis by directly binding to the VaLAR2 promoter and interacting with VaMYBPAR [13]. VvMYC1 depends on the co-expression of VvMYB5a and VvMYB5b induces PA biosynthesis [14]. Thus, genes that induce the transcription of ANR and LAR play crucial roles in the accumulation of flavan-3-ols. However, it is unclear which candidate genes might be involved in salicylic acid (SA)-induced flavan-3-ol biosynthesis.

Salicylic acid directly regulates PA-specific structural genes that affect flavonoid accumulation. Previous studies have suggested that SA is a potent hormonal regulator that induces flavan-3-ol biosynthesis in plants [15–21]. In apple leaves, single and multiple sprays with SA led to an increase in (+)-catechin [20]. SA induced flavan-3-ol accumulation and reduced *Poecilocampa populi* infection in poplar stems [22]. In Chinese cabbage plants, exogenous SA treatment enhanced total flavonoid content by increasing PAL, chalcone isomerase (CHI), flavonol synthase (FLS), and ANS gene expression [23]. Enhanced flavan-3-ol biosynthesis in grape leaves was achieved via stimulation of VvANR transcriptional activity during SA-mediated incubation [24]. Salicylic acid directly or indirectly regulates the *VvPAL* [25], *VvSTS* [26], *VvANR* [24], and *VvLAR* [27] genes to affect the production of phenylpropanoids, PAs, and stilbenes.

Additionally, SA induces the expression of MYBs and the MBW complex. The expression of TFs that activate flavonoid-specific structural genes is upregulated by exogenous SA and stimulates the accumulation of flavonoid compounds. Ullah [28] found that exogenous pretreatment with SA enhanced flavan-3-ol accumulation through activation of the MYB-bHLH-WD40 transcriptional regulatory complex, concurrently exhibiting a suppressive effect on the proliferation of poplar rust. In strawberry 'Benihoppe' leaves, SA pretreatment significantly promoted PA biosynthesis through specific activation of the MBW transcriptional complex and demonstrated effective inhibition of powdery mildew pathogen growth [16]. Our previous studies showed that SA activates the MBW complex (MYBPA1, MYC2, and WDR1) to induce flavan-3-ol production, particularly (+)-catechin and (−)-epicatechin, in grapevines [27].

Although studies have mostly focused on the relationships between SA and flavan-3-ol-specific structural genes, such as *VvPAL*, *VvLAR*, and *VvANR*, little is known about the unknown transcription factors involved in flavan-3-ol biosynthesis as modulated by SA. In this study, two R2R3-MYB TFs that showed positive responses to exogenous SA and were putatively involved in flavan-3-ol biosynthesis were identified through RNA-seq and relevant bioinformatics analysis and qRT-PCR.

2. Materials and Methods

2.1. Plant Materials

Grape berries (*Vitis labrusca*×*vinifera* '8611') were harvested from the horticultural station of Shanxi Agricultural University (112°55′ E, 37°42′ N). The grapevines were positioned with a spacing of 1.0 m within each row and a 2.5 m interval between adjacent rows, aligned in a north–south orientation. Consistent irrigation, soil cultivation practices, pruning techniques, and disease management strategies were implemented for all grape plants. On 18 August 2021, grape berries (clean and healthy with uniform size) were collected from annual branches close to lignification.

2.2. Exogenous SA Treatment

The experiment was performed according to the method of Wen Peng-fei and Chen Jian-ye [25] with modifications. The grape berries (seedless, including skin and flesh) were

washed, dried in the air, and then transferred to equilibrium buffer with SA (150 μM). The equilibrium buffer contained 150 μmol/L SA, 50 mmol/L MES (pH = 5.5), 5 mmol/L $CaCl_2$, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, and 5 mmol/L ascorbic acid. Control grape berries were treated with equal volumes of equilibrium buffer without SA. A pressure of approximately −0.1 MPa for 20 min was used for grape berries in equilibrium buffer. After reversing the pressure, the berries were kept in the media for another 30 min. The samples (CK-0h) were frozen in liquid nitrogen and stored at −80 °C and other samples were subsequently placed in a constant temperature incubator at 25 °C. They were harvested at 30 min and 1 h and then immersed in liquid nitrogen and maintained at −80 °C. All grape berry samples were collected in triplicates and marked as CK-0h-1, CK-0h-2, CK-0h-3, SA-30min-1, SA-30min-2, SA-30min-3, SA-1h-1, SA-1h-2, and SA-1h-3 for RNA-seq analysis. For the extraction of total RNA, qRT-PCR, and measurements of flavan-3-ols, including (−)-epicatechin and (+)-catechin contents (using the CK-0h, SA-30min, and SA-1h samples), three technical replicates were assessed for each biological replicate.

2.3. Content Determination of Flavan-3-Ols

The contents of flavan-3-ols for SA-treated grape berries at 0 h, 30 min, and 1 h were determined using a modified version of the method described by Teng [29]. The grape berries were powdered in liquid nitrogen. Grape berry samples (1.0 g) were immersed in 5 mL of 2% HCl-methanol solution and oscillated at 25 °C and 300 rpm for 24 h in the dark (ZD-85AD, Foshan North and South Chao Electronic Commerce Co., Ltd., Foshan, China). After centrifugation at 12,000 rpm for 10 min (HC-2518R, Haitian Youcheng Technology Co., Ltd., Beijing, China), 100 μL of the supernatant was added to each new 5 mL tube. A total of 100 μL of 2% HCl-methanol solution was added to the tube as the reference solution. Subsequently, 3 mL of 0.1% cinnamic aldehyde (p-DMACA) HCl-methanol solution (1 mol/L) was added. A drop of glycerin was added, mixed, and incubated at room temperature for 10 min. Visible absorbance was measured at 640 nm using 2% HCl-methanol solution as a blank (UV-2600i, Shimadzu Corporation, Kyoto, Japan). The results were expressed as (+)-catechin equivalent (mg/100 g).

2.4. Content Determination of (−)-Epicatechin and (+)-Catechin

High-performance liquid chromatography (HPLC) was used to evaluate the impact of SA on the biosynthesis of grape-derived (+)-catechin and (−)-epicatechin. The contents of (+)-catechin and (−)-epicatechin for SA-treated grape berries at 0 h, 30 min, and 1 h were determined as detailed by Liang [24,30].

2.5. Transcriptome Sequencing Analysis

The samples (CK-0h-1, CK-0h-2, CK-0h-3, SA-30min-1, SA-30min-2, SA-30min-3, SA-1h-1, SA-1h-2, and SA-1h-3) were sequenced at Sangon Biotech Co. (Shanghai, China). Total RNA was extracted using a Total RNA Extractor (TRIzol, B511311, Sangon Biotech Engineering Co., Ltd., Shanghai, China). The RNA concentration and purity were determined using a Qubit2.0 RNA Kit (Life, Q32855, Thermo Fisher Scientific, Waltham, MA, USA). The integrity of the RNA samples was evaluated by 1% agarose gel electrophoresis. Sequencing libraries were prepared using the VAHTSTM mRNA-seq V2 Library Prep Kit (NR601-02; Vazyme, Shanghai Jinpan Biotechnology Co., Ltd., Shanghai, China), specifically designed for Illumina platforms.

2.6. Bioinformatics Analysis

Differentially expressed genes (DEGs) between SA-30 min vs. CK-0 h, SA-1 h vs. CK-0 h, and SA-1 h vs. SA-30 min samples were filtered by DESeq2 using the criteria of absolute log2 (fold change) ≥ 1 and an adjusted p-value ≤ 0.01. Subsequently, DEGs were analyzed using GO, Clusters of Orthologous Groups, and KEGG enrichment.

2.7. Weighted Gene Co-Expression Network Analysis

To analyze the regulatory mechanism of flavonoid biosynthesis and explore the possible SA-responsive sub-genes involved in flavan-3-ol biosynthesis, the WGCNA database was used to construct weighted gene co-expression networks and sub-gene modules [31]. Sub-gene modules and co-expression networks associated with flavonoid metabolism under SA treatment were analyzed by WGCNA (http://bioinformatics.sdstate.edu/idep/, accessed on 12 December 2022). The KEGG pathways associated with genes in each submodule were further analyzed. There were at least 10 genes in each co-expression network. The genes in the flavonoid biosynthetic pathway were further submitted for expression heatmap and co-expression network analysis.

2.8. Quantitative Real-Time PCR

Total RNA was obtained from grape berries (CK-0 h, SA-30 min, and SA-1 h samples) using the improved cetyltrimethylammonium bromide method of Vashisth [32] and then reversed using a PrimeScriptTM RT Reagent Kit with gDNA Eraser (RR047A, Takara Biotechnology Co., Ltd., Beijing, China). The cDNA was diluted to 100 ng/μL. qRT-PCR was performed using a TaqMan One Step RT-qPCR Kit (T2210, Solarbio Biotechnology Co., Ltd., Shanghai, China). To validate the RNA-Seq data, ten genes of interest (*PAL*, *DFR*, *ANR*, *LAR*, *MYBA2*, *MYBA3*, *MYB145*, *MYB108B*, *MYB14*, and *MYB15*) were selected for validation. The expression levels were quantified using the $2^{-\Delta\Delta CT}$ method with grapevine UBQ as an internal control [33]. Primers were designed using Primer Premier 5 software (Table S1).

2.9. Bioinformatic Analysis

A total of 12 MYB protein sequences and 2000 bp upstream of the start codon were obtained using TBtools software 1.0 [34] from the RNA-seq reference genome of *Vitis vinifera*. A phylogenetic dendrogram was constructed using the amino acid sequences of 12 MYB proteins from *Vitis vinifera*, employing the neighbor-joining algorithm in MEGAX software (version 10.2.6). The Cis-elements in the promoters of the 12 *MYB* genes were identified using PlantCARE. The 12 *Vitis vinifera* MYB proteins were confirmed to be R2R3-MYB protein sequences based on the NCBI CDD. The motifs of 12 *Vitis vinifera* MYB proteins were obtained using the online MEME program.

3. Results

3.1. Effect of SA on the Accumulation of Flavan-3-Ols in Grape Berries

To investigate the effect of SA on flavan-3-ol biosynthesis, the grape berries were treated with 150 μM SA. The content of flavan-3-ols increased to 53.8 from 42.2 mg/100 g FW in the control group at 30 min after SA treatment. The total flavan-3-ol content increased by approximately 46.4% with 63.7 mg/100 g fresh weight (FW) at 1 h after SA treatment compared to the control (43.5 mg/100 g FW). SA at 150 μM had a clear influence on the biosynthesis of flavan-3-ols (Figure 1A).

Furthermore, HPLC analyses showed that the level of (+)-catechin started to rise 30 min after SA treatment, increasing to 50.8 mg/100 g FW 1 h after treatment. The content of (−)-epicatechin increased by 19.2% at 1 h after SA treatment (Figure 1B–C).

Next, we focused on the expression levels of genes associated with the production of flavan-3-ols and found *PAL*, *DFR*, *LAR*, *ANS*, and *ANR* to be increased in grape berries treated with SA. Furthermore, *PAL*, *DFR*, *ANS*, and *LAR* were significantly upregulated (2.6-fold, 1.8-fold, 1.7-fold, and 4.2-fold, respectively) compared to the control (Figure 1D–H).

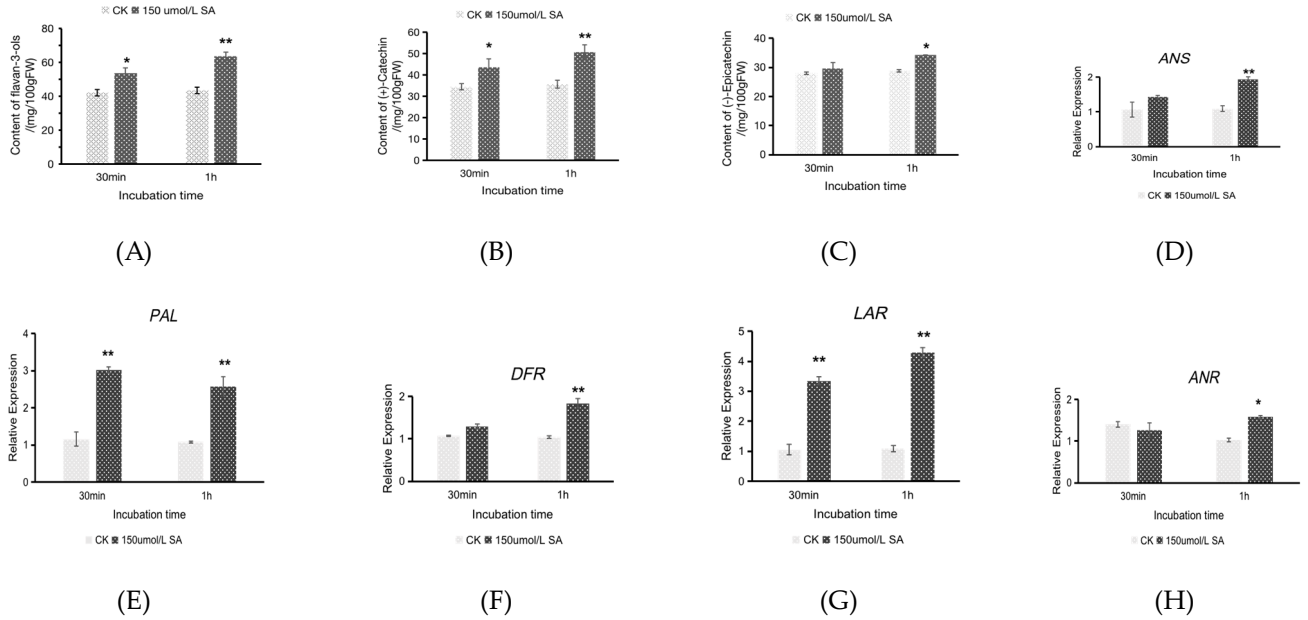


Figure 1. Effect of SA on flavan-3-ol content. (**A**): Total flavan-3-ol content. (**B**,**C**): Content of (+)-catechin and (−)-epicatechin in SA-treated grape berries and control. (**D**–**H**): Expression of structural genes related to flavan-3-ol biosynthesis. HPLC analyses of (+)-catechin and (−)-epicatechin in SA-treated grape berries and control. *PAL*, *DFR*, *ANS*, *LAR*, and *ANR* transcript levels were determined by real-time quantitative PCR normalized relative to the expression of *VvUBQ*. The data are shown as the mean ± SD (with n = 3 biologically independent replicates, * $p < 0.05$, ** $p < 0.01$). Abbreviations: *PAL*, phenylalanine ammonia-lyase; *DFR*, dihydroflavonol 4-reductase; *ANR*, anthocyanidin reductase; *ANS*, anthocyanidin synthase; *LAR*, leucoanthocyanidin reductase; *UBQ*, ubiquitin 1.

3.2. RNA-Seq Analysis

To further explore the mechanism of SA-induced flavan-3-ol biosynthesis and related candidate genes, RNA-seq analysis was performed on grape berries treated with SA for 30 min and 1 h, and on SA-untreated grape berries as the control group. The RNA-seq data revealed a base count range of 4.84G–5.93G per sample, resulting in a total of 48.526G clean bases. The proportion of reads mapped to the reference genome varied from 84.64% to 87.26%, with a unique mapping rate of 82.05–84.47%. The probabilities of mismatches being less than 0.01 and 0.001 were above 97.92% and 93.36%, respectively, while the GC content remained stable at approximately 48–49%, close to the ideal value of 50% (Table 1).

Table 1. Summary statistics of the sequencing data.

Sample Name	Clean Bases	Raw Reads	Clean Reads	Total Mapped (%)	Uniquely mapped (%)	Q20 (%)	Q30 (%)	GC Content (%)
CK-0h-1	5.12G	39,514,172	37,107,302	85.39%	82.77%	97.92%	93.36%	48.96%
CK-0h-2	5.34G	42,240,304	39,285,176	86.60%	83.96%	98.01%	93.57%	48.94%
CK-0h-3	5.22G	40,185,008	37,644,460	87.26%	84.47%	97.99%	93.53%	48.51%
SA-30min-1	5.33G	40,865,788	38,281,884	84.64%	82.05%	97.98%	93.52%	48.68%
SA-30min-2	5.90G	44,632,098	41,909,894	84.83%	82.15%	98.11%	93.81%	48.39%
SA-30min-3	5.68G	43,354,984	40,580,254	84.93%	82.28%	97.98%	93.5%	48.42%
SA-1h-1	5.93G	45,456,646	42,441,890	86.41%	83.69%	98.04%	93.6%	48.33%
SA-1h-2	5.18G	39,979,000	37,408,212	85.19%	82.59%	98.04%	93.69%	49.12%
SA-1h-3	4.84G	37,382,772	35,032,976	85.13%	82.63%	98.07%	93.73%	48.59%

Correlation analysis revealed that the correlation between the three replicates in each group exceeded 0.98, indicating reliability of the results (Figure S1). Principal component

analysis was performed, which revealed greater differences among treatments than among replicates (Figure 2).

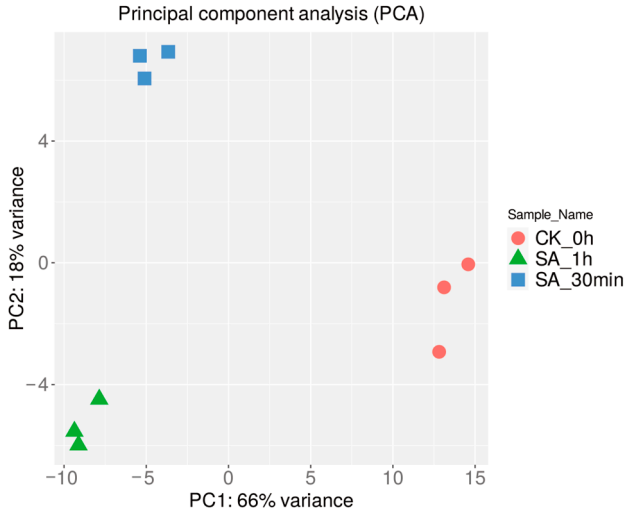
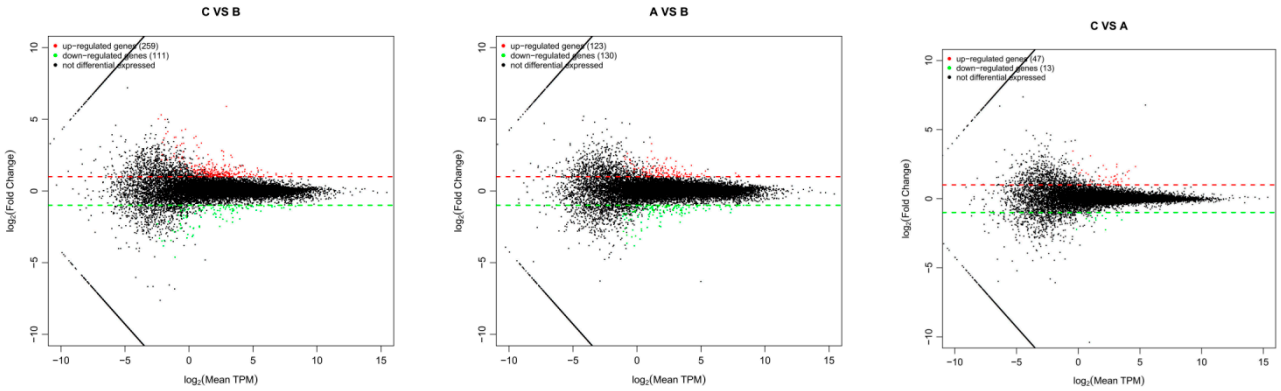


Figure 2. PCA analyses of 9 RNA-seq samples; different shapes represent different materials.

3.3. Differential Expression Gene Analysis

In this study, a gene with a Q value $\leq$ 0.01 and $|\log2FC| \geq 1$ was identified as a DEG. In total, 683 DEGs were screened from the three control groups. To assess the differences in DEGs in various treatments, we created MA maps of DEGs in the SA-1h vs. CK-0h, SA-30min vs. CK-0h, and SA-1h vs. SA-30min groups. Among the DEGs, 370 were identified in the SA-1h vs. CK-0h group, with 259 upregulated and 111 downregulated genes (Figure 3A). In the SA-30min vs. CK-0h group, 253 DEGs were identified, of which 123 genes were upregulated and 130 were downregulated (Figure 3B). In the SA-1h vs. SA-30min group, 60 DEGs were identified, of which 47 genes were upregulated and 13 were downregulated (Figure 3C).

Six clusters were observed among the 434 frequent DEGs (Figure 3D–I). The expression trend for cluster D was an increase in the SA-treated group and a decrease in the untreated group, while the expression trend for cluster G was a decrease in the SA-treated group and an increase in the untreated group. The expression trends in clusters D, E, and F exhibited relatively minor disparities. The overall changes in expression trends in clusters G, H, and I were not statistically significant.



(**A**) (**B**) (**C**)

Figure 3. *Cont.*

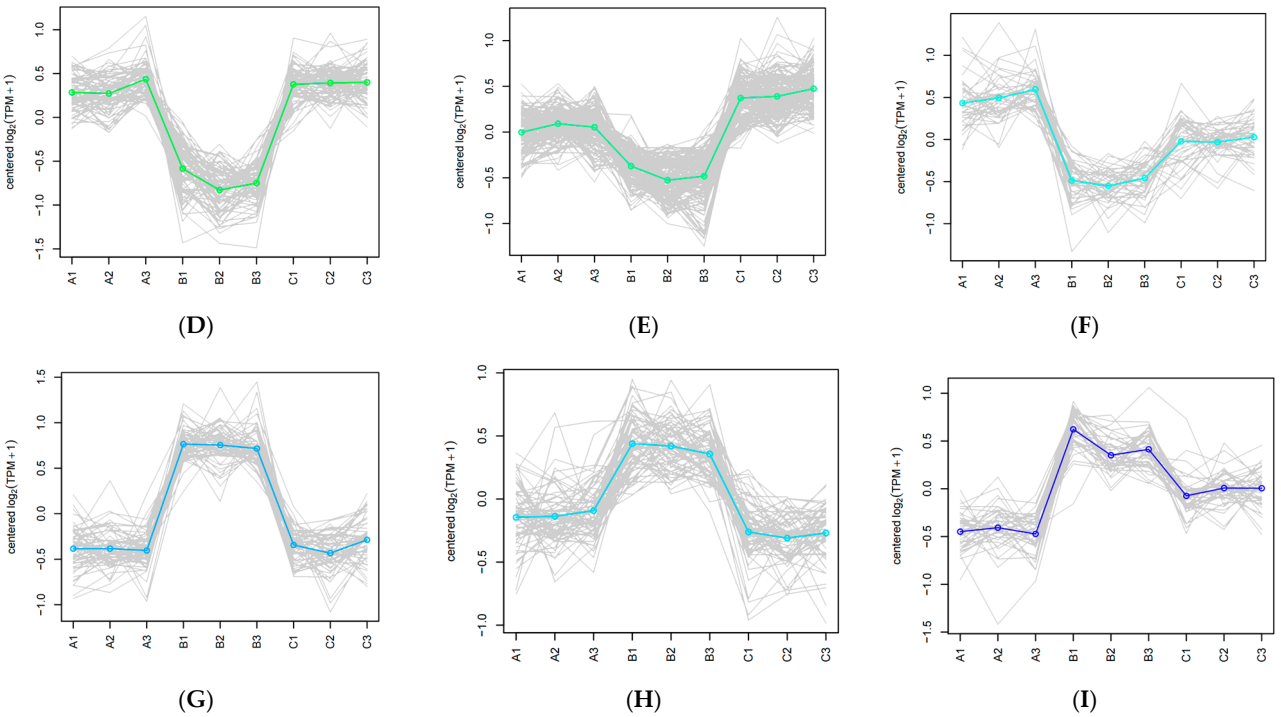


Figure 3. (**A**–**C**): MA plots of DEG responses to SA treatment. Abbreviations: C vs. B, SA-1h vs. CK-0h; A vs. B, SA-30min vs. CK-0h; C vs. A, SA-1h vs. SA-30min. Red dots represent upregulated genes, green dots represent downregulated genes, and black dots represent non-DEGs. (**D**–**I**): Line chart of DEG expression patterns. Abbreviations: B1, CK-0h-1; B2, CK-0h-2; B3, CK-0h-3; A1, SA-30min-1; A2, SA-30min-2; A3, SA-30min-3; C1, SA-1h-1; C2, SA-1h-2; C3, SA-1h-3. Colored lines represent the average expression level of a group of genes.

3.4. Functional Enrichment Analysis

Furthermore, 429 upregulated and 254 downregulated genes in grape berries were analyzed to gain insights into the mechanism of SA-induced flavan-3-ol biosynthesis. For GO analysis, the DEGs were sorted into cellular components, biological processes, and molecular activities.

In the SA-1h vs. CK-0h group, the significantly enriched GO terms included 'DNA binding transcription factor activity', 'transferase activity acyl groups other than amino-acyl groups', 'regulation of transcription DNA-templated', and 'oxidation-reduction process'. Moreover, in the SA-30min vs. CK-0h group, 'transcription regulator activity', 'DNA binding transcription factor activity', 'regulation of nucleic acid-templated transcription', and 'regulation of transcription DNA-templated' were also found to be enriched, which are generally associated with SA. However, the comparison between the SA-1h and SA-30min groups revealed the enrichment of items related to 'the regulation of hormone levels' and 'galactose metabolic processes'. The results suggest that the application of SA to grape berries not only modulates hormone levels in grape berries, but also governs transcriptional activity, DNA-binding transcription factor activity, and oxidation–reduction processes (Figure 4).

KEGG pathway enrichment analysis of the DEGs in the SA-1h vs. CK-0h group revealed enrichment in 'biosynthesis of secondary metabolites' and 'flavone and flavanol biosynthesis'. In the SA-30min vs. CK-0h group, DEGs were enriched in 'phenylpropanoid biosynthesis' and 'flavonoid biosynthesis'. In the SA-1h and SA-30min groups, KEGG pathway enrichment analysis showed enrichment only for 'plant hormone signal transduction' (Figure 5).

Figure 4. GO functional enrichment of DEG responses to SA treatment.

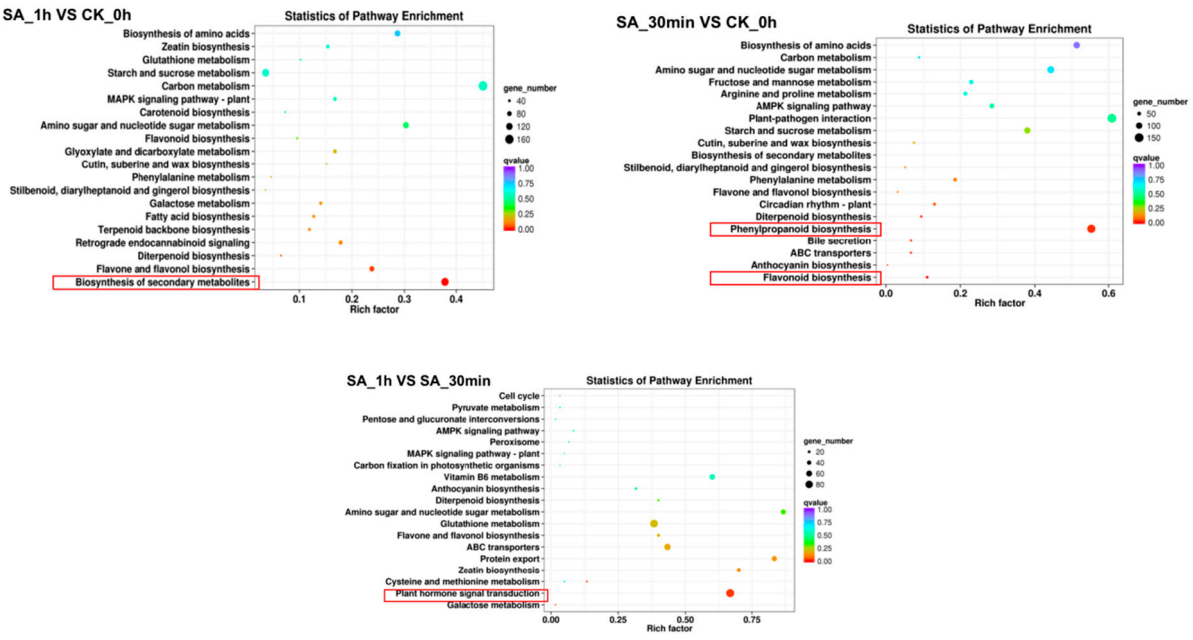


Figure 5. KEGG pathway enrichment of DEG responses to SA treatment.

3.5. WGCNA Exploration of SA-Responsive Genes Involved in Flavan-3-Ol Biosynthesis

To better analyze the SA-responsive candidate factors involved in flavan-3-ol biosynthesis, WGCNA was performed on the 683 DEGs between the three comparison groups.

WGCNA predicts regulatory relationships between genes using expression correlations between genes. To construct a co-expression network associated with flavan-3-ol biosynthesis in grapes, the β soft thresholding parameter was set to 8, scale-free $R^2 > 0.80$, resulting in four gene co-expression modules, with turquoise, blue, brown, and yellow modules. Each branch of the cluster tree represents a module. Each leaf represents a single gene and each color represents a module (Figure 6A).

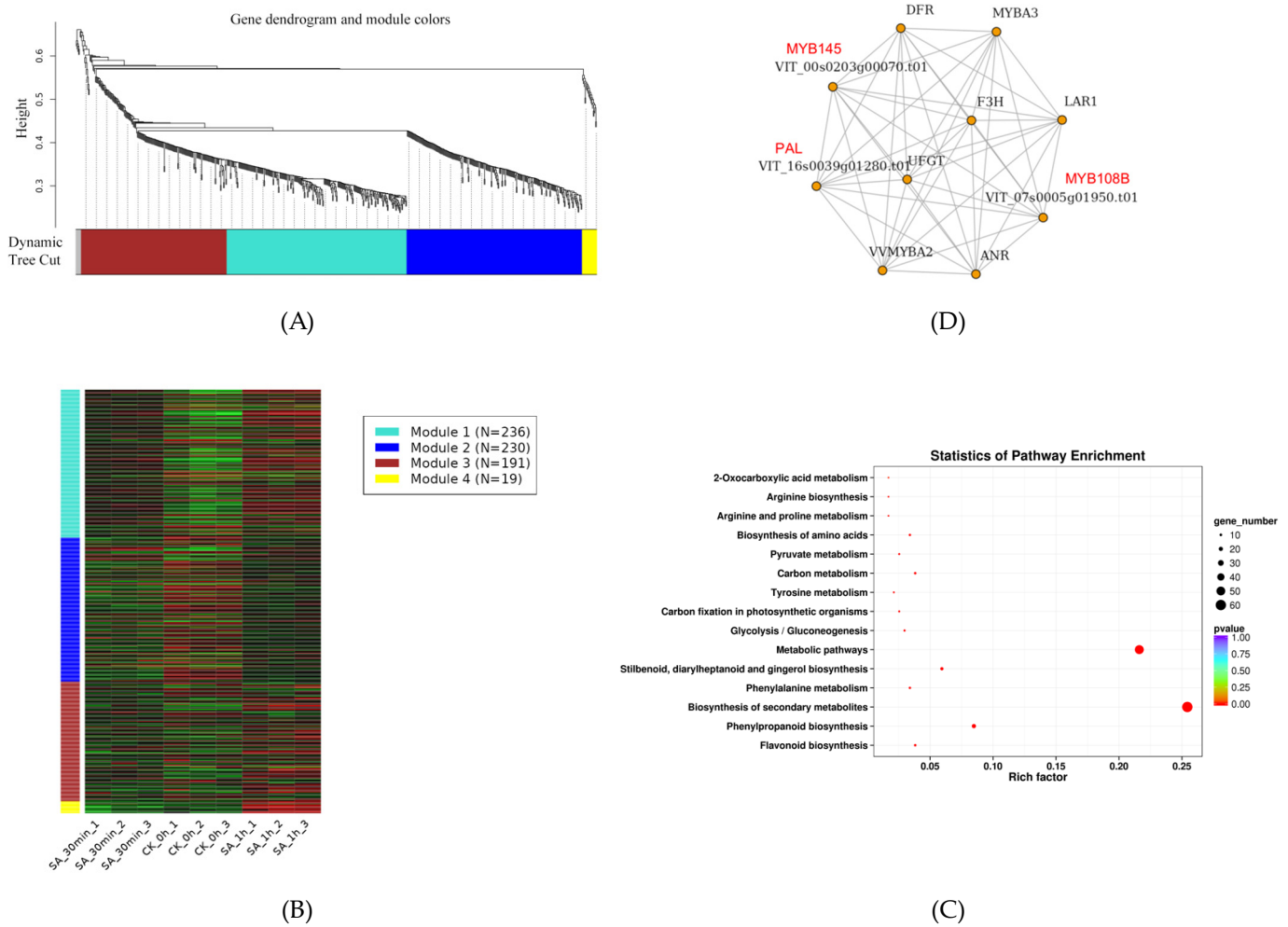


Figure 6. Identification and co-expression analysis of genes probably involved in flavan-3-ol biosynthesis in SA-treatment grape berries using WGCNA. (**A**): Gene dendrogram and module colors. (**B**): Hierarchical clustering of the 683 DEGs by k-Means. (**C**): KEGG pathway enrichment of the 236 DEGs in the turquoise module. (**D**): Identification of a gene interaction co-expression network (turquoise module) using WGCNA.

The maximum number of genes in the turquoise module was 236, followed by the blue module with 230 genes, brown module with 191 genes, and yellow module with 19 genes (Figure 6B). The DEGs in the turquoise module were enriched in 'metabolic pathways', 'biosynthesis of secondary metabolites', 'phenylpropanoid biosynthesis', and 'flavonoid biosynthesis', which are generally related to flavan-3-ol biosynthesis (Figure 6C). Ten hub genes were identified in the gene interaction network of turquoise modules. These were *PAL*, *F3H*, *DFR*, *UFGT*, *ANR*, *LAR*, and four MYB family genes (*MYB145*, *MYB108B*, *MYBA2*, *MYBA3*), which may be involved in flavan-3-ol biosynthesis as modulated by SA (Figure 6D).

3.6. TF Expression Analysis

The expression patterns of *MYB* genes are displayed using heatmaps. The TFs (*MYB145* and *MYB108B*) revealed the highest expression in the SA-1h vs. CK-0h group (Figure 7A,B).

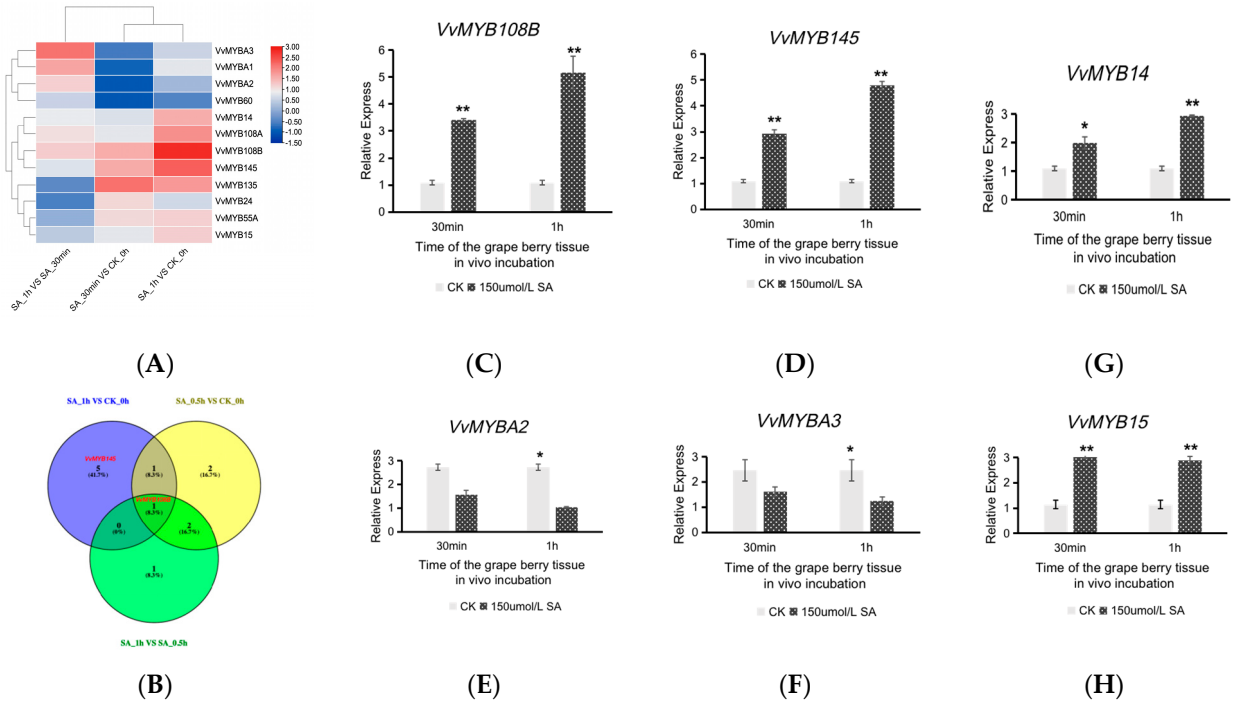


Figure 7. (**A**): The expression of MYB responses to SA treatment. FPKM of MYB-DEG responses to SA treatment. (**B**): MYB-DEGs Wayne diagram. (**C**–**F**): Four hub genes responding to SA treatment and involved in flavan-3-ol biosynthesis transcript level were determined by real-time quantitative PCR normalized relative to the expression of *VvUBQ*. (**G**, **H**): Two identified gene transcript levels were determined by real-time quantitative PCR to confirm the reliability of the RNA-seq data. The data are shown as the mean ± SD (with n = 3 biologically independent replicates, * $p < 0.05$, ** $p < 0.01$). Abbreviations: FPKM, fragments per kilobase of exon model per million reads mapped; *UBQ*, ubiquitin 1.

The mRNA expression levels of the four hub genes in various materials were assessed using qRT-PCR (Figure 7C–F). Among the four genes, *MYB145* and *MYB108B* exhibited higher expression in SA-treated samples, indicating their positive regulatory relationships with SA (Figure 7C,D), while two genes (*MYBA2* and *MYBA3*) showed lower expression in SA-treated samples, implying their negative regulatory relationships with SA (Figure 7E,F). Therefore, we identified two candidate genes (*MYB145* and *MYB108B*) modulated by SA and putatively involved in flavan-3-ol biosynthesis through RNA-seq, relevant bioinformatics analysis, and qRT-PCR. These findings lay a solid basis for a more profound understanding of the molecular regulation of flavan-3-ol biosynthesis in grapes.

The qRT-PCR results of six genes (*MYB145*, *MYB108B*, *MYBA2*, *MYBA3*, *MYB14*, and *MYB15*) were also consistent with the RNA-seq results (Figure 7C–H).

3.7. Motif Composition and TCA-Elements of the 12 MYB Genes

All 12 significantly different *MYB* genes were checked to determine the presence of MYB DNA-binding structural domains. The potentially conserved motifs in the 12 MYB proteins were investigated using MEME 5.5.7 online analysis software. Ten motifs were found in the 12 MYB proteins. Twelve MYB sequences containing motifs 1, 2, and 3 matched the characteristics of *R2R3-MYB* genes (Figure 8A).

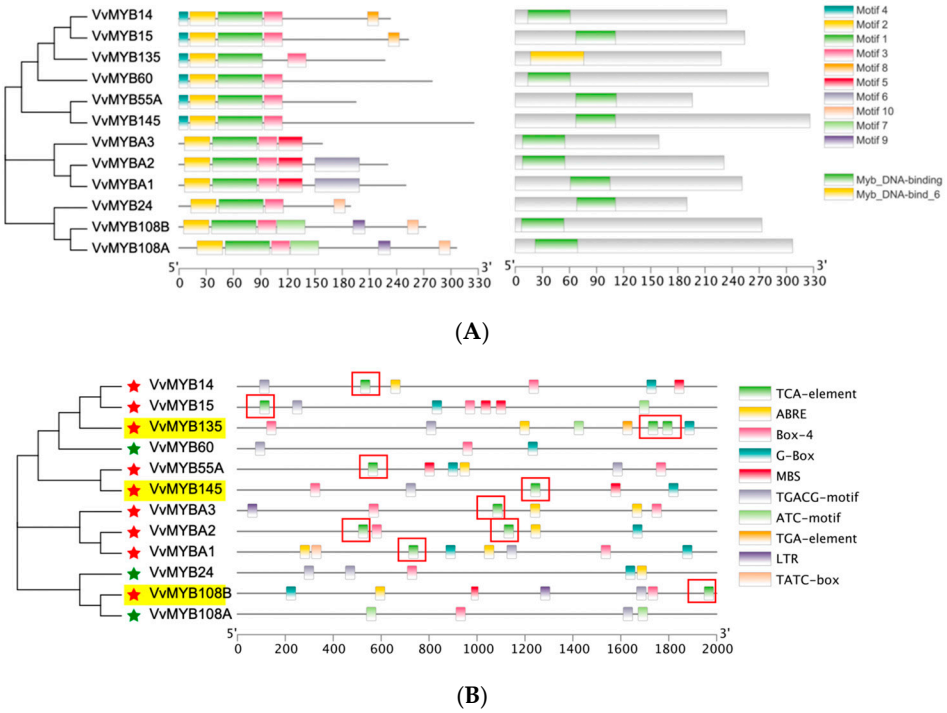


Figure 8. (**A**): Conserved motifs, conserved domains analysis of 12 MYB-DEGs. (**B**): Predicted *cis*-elements analysis of 12 MYB-DEGs. The red star denotes TCA elements are present in the promoter regions of 9 R2R3-MYB family genes. The green star denotes TCA elements are not present in the promoter regions of 3 R2R3-MYB family genes. The yellow background denotes promoter regions within 800 bp of the coding sequence. Abbreviations: TCA elements, tobacco catabolic acid elements.

Promoter *cis*-elements hold important influences in the process of gene expression. Tobacco catabolic acid (TCA) elements involved in the SA response were present in the promoter regions of 12 *R2R3-MYB* family genes, in addition to *VvMYB60*, *VvMYB24*, and *VvMYB108A*. Notably, TCA elements were detected in the *VvMYB145*, *VvMYB108B*, and *VvMYB135* promoter regions within 800bp of the coding sequence. Thus, these *R2R3-MYB* genes may be controlled by SA in grape berries (Figure 8B).

4. Discussion

4.1. Exogenous SA Treatment Significantly Promotes PAL, DFR, and LAR Expression and Flavan-3-Ol Accumulation in Grapevines

Salicylic acid is a phenolic phytohormone that is considered a key signaling molecule involved in stimulating the accumulation of PAs. Wei et al. found that 100 mg/L exogenous SA in young, color-changing, and ripening 'Jizaomi' grapevine berries could enhance flavan-3-ol accumulation [35]. Exogenous SA induced (+)-catechin, (−)-epigallocatechin and (−)-epicatechin biosynthesis in 'Zaoheibao' grapevine leaves [27]. Wen et al. reported that 150 µM exogenous SA induced PA biosynthesis in vivo in incubated grape berry tissues [25]. In this study, we observed the effects of 150 µM exogenous SA on flavan-3-ol biosynthesis and found that SA treatment significantly promoted flavan-3-ol accumulation in grapevine berries at 30 min and 1 h (Figure 1A). In *Vitis vinifera* cell suspension cultures, treatment with exogenous SA enhanced the production of PAs and anthocyanins, and induced the expression of *VvCHS*, *VvCHI*, *VvDFR*, and *VvANS* [36]. In this study, exogenous SA directly regulated PA-specific structural genes (*VvPAL*, *VvDFR*, *VvLAR*, *VvANS*, and *VvANR*) to affect flavan-3-ol accumulation, which is consistent with the findings of Chen and Yang [24,25]. Thus, the treatment of grapevines with an appropriate concentration of exogenous SA promotes *PAL*, *DFR*, and *LAR* expression and the accumulation of flavan-3-ols, such as (−)-epicatechin and (+)-catechin. Further studies are necessary to explore the

unknown transcription factors likely involved in flavan-3-ol biosynthesis as modulated by exogenous SA.

4.2. Two Candidate Genes (VvMYB108B and VvMYB145) Responded to Exogenous Salicylic Acid Treatment and Probably Induced Flavan-3-Ols Biosynthesis

Recently, numerous flavan-3-ol biosynthesis-related TFs have been discovered in plants that induce the transcription of ANR and LAR, which play an important role in the accumulation of flavan-3-ols. In the leaf tissues of *Lotus* spp., a complex consisting of the MYBPA2, TT2b, and TT8 proteins acts as a key regulator of *ANR* and *LAR* gene expression, which in turn promotes PA production [37]. In grapevines, *VvWRKY24* regulates PA synthesis by binding to the promoter sequences of DFR and LAR [38]. In juvenile grapevine fruits, *VvMYBPA1* specifically recognizes and binds to the promoter regions of *VvLAR* and *VvANR*, as well as promotes the accumulation of flavane-3-ols [30]. In *Ginkgo biloba*, 2991 DEGs were detected between the control and SA treatment groups using RNA-seq analysis [39]. Several MYB proteins were overexpressed in the SA treatment group. In this study, RNA-seq analysis revealed 683 DEGs in the three control groups (SA-1h vs. CK-0h, SA-30 min vs. CK-0h, and SA-1h vs. SA-30min). These DEGs responded strongly to exogenous SA treatment. The DEGs of the WRKY, MYB, WD40, and bHLH transcription factors were identified in SA-treated grapevine berries. However, the candidate genes for SA-induced flavan-3-ol accumulation in grapevine berries remain unknown. The WGCNA analysis was conducted to identify candidate genes that responded to exogenous SA treatment and probably induced flavan-3-ol biosynthesis.

In grape suspension cells, WGCNA revealed that *VvCORA-like* potentially regulates phenolic compound biosynthesis and metabolic pathways under salinity stress [40]. In apricot fruits, 26 candidate genes involved in flavonoid metabolite biosynthesis were identified using WGCNA [41]. Furthermore, WGCNA was used to identify 12 candidate genes associated with flavonoid biosynthesis in blueberry calli [42]. In the present study, using WGCNA, four candidate genes (*VvMYBA2*, *VvMYBA3*, *VvMYB108B*, and *VvMYB145*) likely related to flavan-3-ol biosynthesis were identified in SA-treated grapevine berries. In grapevines, *VvMYBA2* [43], *VvMYBA3* [44] promote anthocyanin biosynthesis. *VvMYB108B* and *VvMYB145* have not been reported. These two candidate genes (*VvMYB108B* and *VvMYB145*) may participate in PA biosynthesis and may be essential for SA-induced flavan-3-ol biosynthesis, thus providing a basis for studying the potential mechanisms of SA-induced flavan-3-ol synthesis in grapevines. The qRT-PCR analysis indicated that *VvMYB108B* and *VvMYB145* were upregulated in the SA-treated grapevine berries. In grapevines, these two genes (*VvMYB108B* and *VvMYB145*) respond to exogenous SA treatment and putatively act as potential regulators of flavan-3-ol production.

5. Conclusions

The results revealed that 683 DEGs were identified in response to exogenous SA treatment using RNA-seq, and two candidate genes (*VvMYB108B* and *VvMYB145*) putatively participating in flavan-3-ol biosynthesis were selected by constructing a co-expression network and using qRT-PCR. However, the relationship between SA-modulated flavan-3-ols-specific structural genes (*VvLAR* and *VvANR*) and these two candidate genes remains unclear and will be the focus of our future research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14112069/s1, Figures S1–S9: Correlation analysis between the three replicates in each group; Table S1: qRT-PCR primer design.

Author Contributions: F.Y. coordinated the study, obtained the results, and wrote the manuscript. W.G. determined the contents of flavan-3-ol monomers of grape fruits at different stages treated with salicylic acid P.Z. installed the software and processed the data. R.Z. and S.Z. determined the contents of (−)-epicatechin and (+)-catechin. J.L. and Y.G.G. supervised the study and drafted the manuscript. P.W. and F.Y. designed and supervised the study, interpreted the data, and drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

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