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Integrated Analysis of Transcriptome and Metabolome Provides Insights into Phenylpropanoid Biosynthesis of Blueberry Leaves in Response to Low-Temperature Stress

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Abstract

The phenylpropanoid compounds are crucial secondary metabolites for blueberry plants. Low temperatures induce the expression of phenylpropanoid biosynthesis genes and regulate the accumulation of phenylpropanoid metabolites. However, the molecular mechanisms of blueberry leaves in response to low-temperature stress are unknown. To explore the molecular mechanisms of phenylpropanoid biosynthesis under low-temperature stress, the 6-month-old blueberry plants were cultured at 10 °C for 0, 6, 12, 24, and 48 h. The total of 16,388 differentially expressed genes (DEGs) and 303 differentially accumulated metabolites (DAMs) were identified by transcriptome deep sequencing (RNA-seq) and ultra-high performance liquid mass spectrometry, respectively. The most enriched low-temperature-responsive genes are mainly involved in the phenylpropanoid biosynthesis pathway and the main low-temperature-responsive metabolites come from the phenylpropanoid superclass based on transcriptome and metabolome data, respectively. *CBF2* plays essential roles in the ICE-CBF-COR regulatory pathway, and transcription factors (TFs) *ERF109*, *MYB14*, *WRKY40*, *HSP30*, *MPSR1*, *ZHD4*, *MADS3*, and *MADS27* might be responsible for blueberry leaf low-temperature tolerance. The MYB TFs from group 5, group 6, and group AtMYB5 may regulate the accumulation of phenylpropanoid metabolites by regulating expression of phenylpropanoid biosynthesis genes. These findings uncover possible molecular mechanisms of phenylpropanoid biosynthesis during low-temperature stress and provide a basis for future studies and crop improvement.

Keywords: RNA-seq; UHPLC-MS/MS; differentially expressed genes; differentially accumulated metabolites; transcription factors; MYB family

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1. Introduction

Low-temperature stress is a crucial environmental factor limiting the distribution of plants, affecting their growth and development, and reducing crop productivity [1,2]. To cope with cold-induced damage, plants have evolved precise and efficient mechanisms, ranging from sensing cold signals to adjusting physiological and biochemical responses [3]. Cold acclimation research has led to a number of important findings in the past decades in which *C-repeat binding factor* (*CBF*) plays essential roles in the cold stress response by binding to the promoters of the *cold-regulated* (*COR*) genes and regulating their expressions. *C-repeat binding factor* (*CBF*) *expression 1* (*ICE*) contributes to the improved cold

tolerance by directly activating the *CBF* genes. Many studies have proved that the *ICE-CBF-COR* regulatory pathway plays a core role in the regulation of low-temperature stress response [4,5]. At the same time, low temperatures regulate phenylpropanoid compound biosynthesis via CBF transcription factors. For example, in ripening litchi fruits, LcDREB2C, a CBF/DREB transcription factor, promoted anthocyanin accumulation by directly binding to the promoters of *LcMYB1*, *LcCHI*, and *LcF3H* at low temperatures [6,7].

Phenylpropanoids are a vast family in the plant kingdom and can improve tolerance low-temperature environments [8,9]. In this family, flavonoids, isoflavonoids, cinnamic acids, and coumarins are the main phenylpropanoid compounds. The phenylpropanoid metabolites are biosynthesized via the general phenylpropanoid pathway, with key steps catalyzed by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate CoA ligase (4CL) enzymes, which subsequently enters the simple phenylpropanoid biosynthetic pathway by O-hydroxycinnamoyltransferase (HCT), cinnamoyl-CoA reductase (CCR), and caffeoyl shikimate esterase (CSE). It enters the flavonoid biosynthetic pathway through chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), flavonoid 3′5′-hydroxylase (F3′5′H), and so on. Then flavonol synthase (FLS) catalyzes flavonol biosynthesis, and 2-hydroxyisoflavanone dehydratase (HID), isoflavone malonyltransferase (IMaT) and isoflavone 2′-hydroxylase (I2′H) catalyze isoflavonoid biosynthesis, while dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) catalyze anthocyanin and flavan-3-ol biosynthesis. The UDP-glucose flavonoid 3-O-glucosyl transferase (UFGT) and UDP-glycosyltransferase (UGT) catalyze the last step in anthocyanin and flavonol glycoside biosynthesis, while anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) are responsible for flavan-3-ol biosynthesis [10]. The genes encoding these enzymes regulate phenylpropanoid accumulation and increase low-temperature tolerance.

Transcription factors (TFs), such as WRKY, MYB, AP2, WRKY, bHLH, and NAC families, may simultaneously control numerous pathways during low-temperature stresses in plants [11–13]. MYB TFs involved in the regulation of cold tolerance have been functionally identified in many species. For *Arabidopsis*, *AtMYB14* and *AtMYB15* are involved in cold regulation in *CBF* genes [14,15]. In apple, *MdMYB23*, *MdMYB4*, *MdMYB88*, *MdMYB124*, and *MdSIMYB1* positively regulate cold tolerance and cold-responsive gene expression [16–18], whereas *MdMYB44* and *MdMYB15L* negatively regulate plant cold tolerance [19,20]. Furthermore, MYB TFs have been characterized to regulate phenylpropanoid metabolism accumulation in response to low-temperature signals. For example, apple *MdMYB308L* was found to regulate cold tolerance and anthocyanin accumulation [21]. *CeMYB52* (*Cymbidium ensifolium*) acts as a key temperature-responsive TF regulating anthocyanin biosynthesis and transport via *CeCHS8* and *CeGST* [22]. *MeMYB2* (*Manihot esculenta* Crantz) appears to act as an inhibitor of chilling-induced anthocyanin accumulation during the rapid response of cassava to chilling stress [23].

Blueberries (*Vaccinium corymbosum* L.) is one of the most economically important fruit crops in China, in which 'Northland' are the main cultivated cultivars in northeast China, and they are severely affected by cold damage [24]. Low temperatures cause significant economic losses in blueberry production. Especially in the early spring in northern China, blueberries often suffer from low-temperature damage, so it is of great significance to improve low-temperature tolerance in the flowering and fruiting stages of blueberries. The leaves and fruits of blueberry are known to be rich in phenylpropanoid compounds for use either as dietary botanicals or by the pharmaceutical industry [25,26]. Transcriptomic analysis revealed that many differentially expressed genes (DEGs) and differentially abundant proteins were mainly involved in the pathways of protein processing in the endoplasmic reticulum, the glutathione metabolism pathway, and ribosomes during overwintering in blueberries [24]. However, the mechanism of blueberry responses to low-temperature

stress is unclear. Transcriptome and metabolome analyses are powerful tools for identifying DEGs and differentially accumulated metabolites (DAMs) under low-temperature and providing more information for the gene-to-metabolite network [27]. Thus, it is important to explore the molecular regulatory mechanisms of low-temperature stress response in blueberry by integrative transcriptomic and metabolomic data for the breeding of low-temperature-resistant blueberry cultivars.

In this study, DEGs were identified in blueberry leaves during low-temperature stress by transcriptome analysis, and we also screened DEGs from *ICE-CBF-COR* regulatory and phenylpropanoid biosynthetic pathways and differentially expressed MYB genes. The metabolomic substances were detected by ultra-high performance liquid mass spectrometry (UHPLC-MS/MS), and differentially accumulated phenylpropanoid metabolites were also identified under low-temperature stress by metabolome data. To elucidate the mechanism driving phenylpropanoid biosynthesis in blueberry leaves under low temperatures, we assembled a regulatory network encompassing phenylpropanoid biosynthetic genes, *MYB* genes, and phenylpropanoid metabolites by integrating transcriptome and metabolome data. The results of this study will broaden our understanding of low-temperature-induced phenylpropanoid metabolite biosynthesis.

2. Materials and Methods

2.1. Plant Materials and Low-Temperature Stress Treatments

The in vitro-grown blueberry (*Vaccinium corymbosum*) cultivar 'Northland' seedlings were cultured on modified woody plant medium (WPM) containing Murashige and Skoog vitamins and 1.0 mg/L trans-Zeatin under a 16/8h light/dark photoperiod at 25 °C in the College of Plant Science of Jilin University, China. In vitro-grown 40 d seedlings were transferred to 7 cm pots containing soil and cultured in the artificial climate chamber in a growth condition of 25 °C temperature, 70% relative humidity, and a 16/8h light/dark photoperiod. The temperature of the chamber was set to 10 °C after 6 months, and the first-to-third fully expanded leaves from randomly selected plants were collected from the 10 °C treatment after 0, 6, 12, 24, and 48 h. The samples were labeled in sequence as L0h, L6h, L12h, L24h, and L48h, rapidly flash-frozen in liquid nitrogen, and stored at −80 °C for transcriptome deep sequencing (RNA-seq) analysis (three independent biological replicates) and metabolomic profiling (five independent biological replicates).

2.2. Transcriptomic Analysis by Transcriptome Deep Sequencing

The RNAs of the samples were obtained using TRIzol reagent (Invitrogen, Walthan, MA, USA) to construct RNA-seq libraries. MGISEQ-T7 (MGI, Shenzhen, China) was used for high-throughput sequencing, and 150 bp paired-end reads were generated. All of the analyses were performed using an in-house pipeline from Shanghai Applied Protein Technology (Shanghai, China). The GC content of clean reads and quality scores of Q20 and Q30 were used to evaluate base quality. The clean reads were compared with the blueberry genome (https://www.vaccinium.org/analysis/49, accessed on 20 April 2025) using HISAT2 software version 2.2.1. The values of fragments per kilobase of transcript per million fragments mapped (FPKM) were used to evaluate gene expression. DESeq2 software version 1.30.1 was used to identify the DEGs by the criteria of absolute $\log_2$ fold change (FC) $\geq$1 and p-value < 0.05. Among them, the values of $\log_2$ (FC) $\geq$ 1 and $\log_2$ (FC) $\leq$ −1 were defined as the up-regulation and down-regulation of gene expression levels, respectively. The Kyoto Encyclopedia of Genes and Genomes (KEGG) tools (Kyoto, Japan) were used to analyze the DEGs.

2.3. RNA Sequencing Data Validation

The samples used for RNA-seq (samples were treated at 10 °C for 0, 6, 12, 24, and 48 h, with three independent biological replicates in each treatment) were employed for confirmation of the accuracy and reliability of the RNA-seq data. The genes of interest, *CBF2*, *ERF109*, *MYB14*, *WRKY40*, *HSF30*, *MPSR1*, *ZHD4*, and *MADS3*, were selected for RT-qPCR analysis, and *GAPDH* (AY123769) was used as the reference transcript. Primer sequences are presented in Table S1. The experiments were carried out with three technical replicates for each independent biological replicate. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of each gene. SPSS 19.0 software was used for Tukey's test, and the p value of ≤ 0.05 was considered a significant difference between treatments.

2.4. Metabolomics Analysis by UHPLC-MS/MS

Each powder sample was added to pre-cooled methanol/acetonitrile/H_2O (2:2:1, $v/v/v$) solution, followed by vortex mixing, low-temperature ultrasonication for 30 min, standing at −20 °C for 10 min, and centrifugation at 14,000 g at 4 °C for 20 min, and the supernatant was vacuum-dried. For mass spectrometry analysis, 0.1 mL of acetonitrile/water (1:1, v/v) was added to re-dissolve the sample, vortexed, and then centrifuged at 14,000× g at 4 °C for 15 min. The supernatant was taken for LC-MS analysis.

The samples were separated using the Agilent 1290 infinity LC ultra performance liquid chromatography (UHPLC) (Santa Clara, CA, USA) with a C-18 column; column temperature: 40 °C; flow rate: 0.4 mL/min; injection volume: 2 μL; mobile phase composition: A: 25 mM ammonium acetate and 0.5% formic acid in water; B: methanol; The gradient elution procedure is as follows: 0–0.5 min, 5% B; within 0.5 to 10 min, B linearly changes from 5% to 100%; from 10.0 to 12.0 min, B remained at 100%; from 12.0 to 12.1 min, B linearly changed from 100% to 5%; 12.1–16 min, B remained at 5%. Quality control (QC) samples were inserted into the sample queue to monitor and evaluate the stability of the system and the reliability of the experimental data.

For analysis of the metabolome data, ProteoWizard MSConvert was used to convert the raw data to the final data format, and MS-DIAL software (ver. 4.60) was used to normalize the matched peak data and peak area data. DAMs were identified from positive and negative ion mode according to fold change >1.5 (upregulation) or fold change <0.67 (down-regulation) and p-value < 0.05. Pareto-scaled principal component analysis (PCA) was carried out by multivariate statistical analysis using the R package ropls version 1.14.11.

2.5. Integrative Analysis of Transcriptomic and Metabolomic Data

The KEGG pathways of phenylpropanoid, flavonoid, flavonol, and anthocyanin were mapped using the above-identified target DEGs and DAMs. The correlations between DEGs and DAMs from phenylpropanoid biosynthesis pathways were calculated by Pearson correlation coefficients using SPSS software. Heatmaps of gene expression levels were constructed using scale-normalized $\log_{10}$ (FPKM), and heatmaps of metabolites were constructed using scale-normalized $\log_{10}$ (peak intensity) by Tbtools (v1.098761) software.

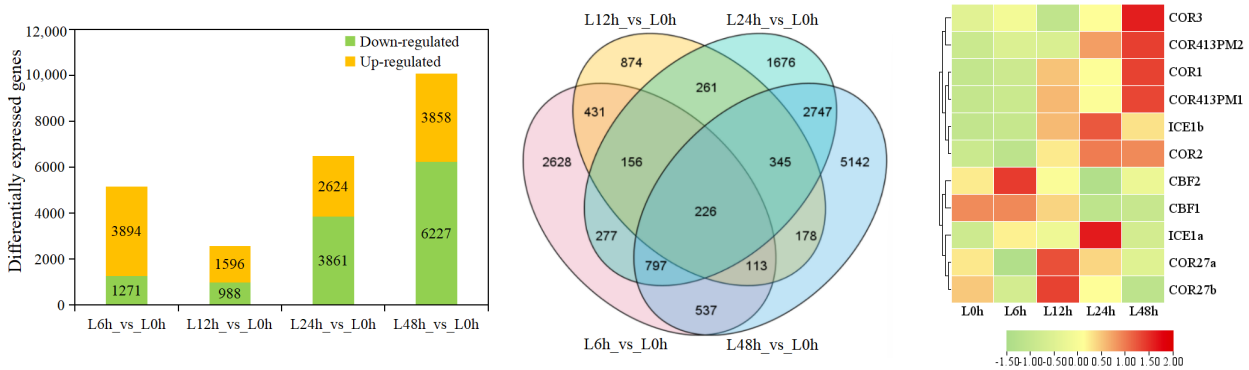
3. Results

3.1. Transcriptome Analysis and Differentially Expressed Genes in Response to Low-Temperature Stress

To investigate the responses of blueberry to low-temperature stress, RNA-Seq technology was employed to perform transcriptome analysis on 15 blueberry samples. In total, 11.94–14.33 Gb of clean bases was obtained for each sample, with a Q30 ranging from 89.09 to 92.40% and a GC content ranging from 46.16% to 46.59%. Here, 89.79–91.54% of all clean reads per sample were successfully mapped to the blueberry reference genome (Table

S2). PCA showed that samples were dispersed between treatments, while samples within treatments were clustered together (Figure S1). These results implied that the sequencing quality was high and the sequencing data met the requirements for further analyses.

A total of 16,388 DEGs were detected between the control samples (0 h) and each time point of the low-temperature stress (Table S3). We detected 5165 DEGs in 6 h (3984 up-regulated and 1271 down-regulated), 2584 in 12 h (1596 up-regulated and 988 down-regulated), 6485 in 24 h (2624 up-regulated and 3861 down-regulated), and 10, 085 in 48 h (3858 up-regulated and 6227 down-regulated) under low-temperature stress compared to the 0 h control samples (Figure 1a). However, the Venn diagram showed that only 226 DEGs were shared for all the pairwise-compared groups (Figure 1b).



(**a**) (**b**) (**c**)

Figure 1. Differentially expressed genes (DEGs) under low-temperature stress in blueberry leaves in response to low-temperature stress identified by transcriptome deep sequencing. (**a**) Number of DEGs. "Down-regulated" and "Up-regulated" represent down-regulated and up-regulated DEGs, respectively. (**b**) Venn diagram showing the extent of overlap between DEGs across pairwise comparisons. (**c**) The heatmap represents the differentially expressed genes from the *ICE*-CBF-COR regulatory pathway. L0h, L6h, L12h, L24h, and L48h represent samples treated with low temperatures for 0, 6, 12, 24, and 48 h, respectively.

To clarify the response of cold-regulatory genes to low-temperature stress, the *ICE-CBF-COR* regulatory pathway genes were screened (Table S4). A total of nine differentially expressed *COR* genes responded to low-temperature stress, in which *COR1* and *COR2* were significantly up-regulated at 12 h, 24 h, and 48 h of low-temperature stress compared to the 0 h control. We also found that *COR3*, *COR413IM1*, *COR413PM1*, and *COR413PM2* were significantly up-regulated; however, *COR27a* and *COR27b* were significantly down-regulated during low-temperature stress, in which *COR1*, *COR2*, and *COR413PM1* were up-regulated more than 3-fold at 48 h of low-temperature stress compared to the 0 h control. *CBF1* and *CBF2* were also induced under low-temperature stress, in which *CBF2* was up-regulated more than 16-fold at 6 h of low-temperature stress compared to the 0 h control. Both *ICE1a* and *ICE1b* were up-regulated at 24 h of low-temperature stress compared to the 0 h control. These genes may form a blueberry *ICE-CBF-COR* regulatory network under low temperatures (Figure 1c). We also found that there are similar change trends between RNA-seq data and RT-qPCR data under low-temperature stress, indicating the accuracy and reliability of the RNA-seq data (Figure S2).

3.2. Functional Annotation and Enrichment Analysis of Differentially Expressed Genes

The KEGG database was used to annotate the functions of the DEGs (Table S5). All the DEGs were assigned to 271 KEGG pathways, of which 104 KEGG pathways were shared in all pairwise comparisons (Figure S3). In the top 20 KEGG enrichment analysis, plant–pathogen interaction, protein processing in the endoplasmic reticulum, plant

hormone signal transduction pathways at 6 h, and metabolic pathway at 12 and 24 h of low-temperature stress compared to 0 h control samples were significantly enriched (Figure 2). We also found that DEGs involved in the phenylpropanoid biosynthesis pathway and anthocyanin biosynthesis pathway were significantly enriched at 24 and 48 h of low-temperature treatment.

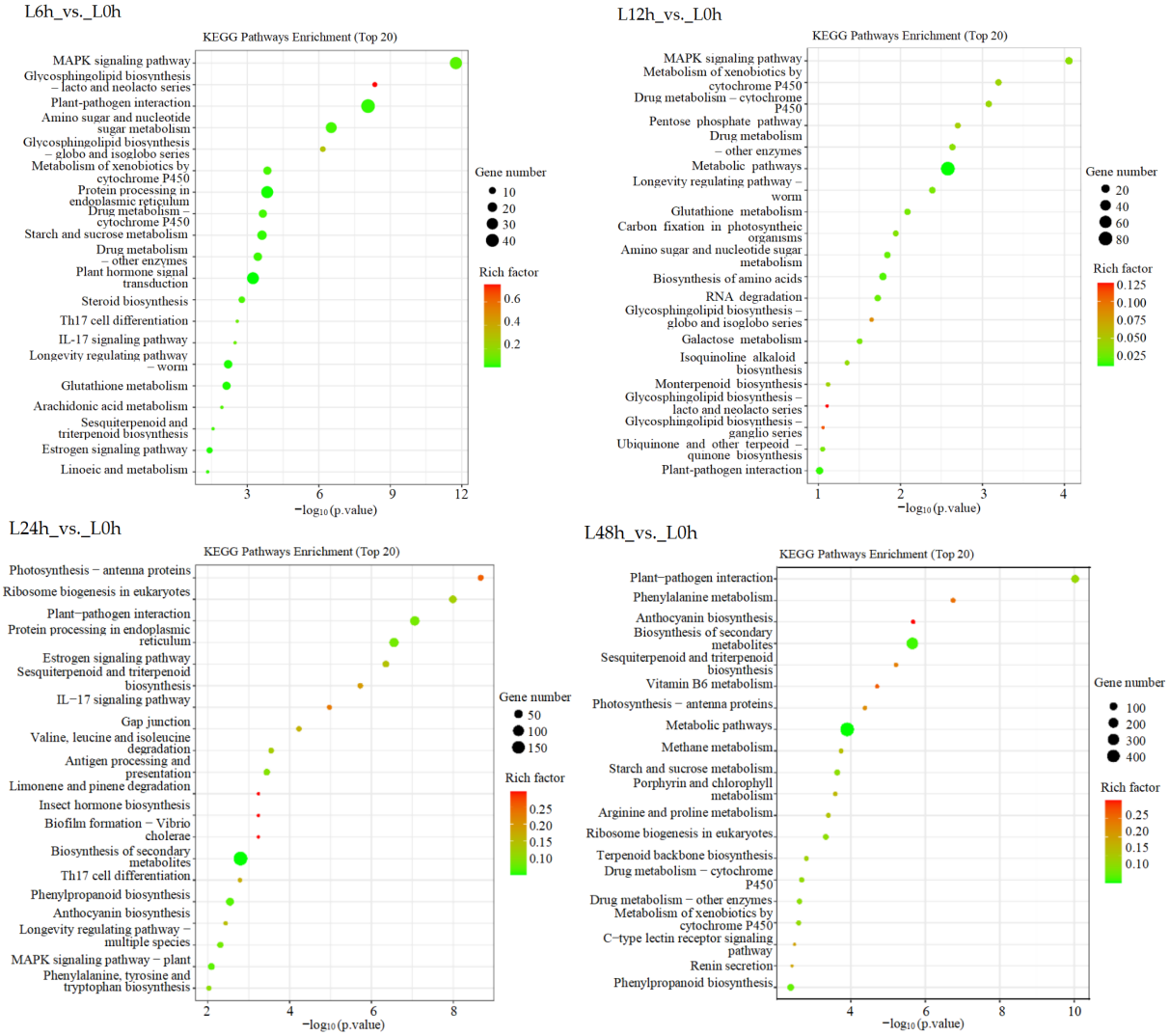


Figure 2. The top 20 KEGG enrichment analysis of differentially expressed genes under low-temperature stress in blueberry leaves identified by transcriptome deep sequencing. L0h, L6h, L12h, L24h, and L48h represent samples treated with low temperatures for 0, 6, 12, 24, and 48 h, respectively.

3.3. The Differentially Expressed Transcription Factors in Response to Low-Temperature Stress

To explore the responses of TFs to low-temperature stress, differentially expressed TFs were screened (Table S6). A total of 1578 differentially expressed transcription factor genes were identified and belonged to 20 major TF families, including zinc finger (27.95%), MYB (11.60%), AP2 (10.77%), WRKY (5.83%), bHLH (4.82%), NAC (4.37%), and so on (Figure 3a). Among them, the expression levels of *CBF2* and *ERF109* (AP2 family), *MYB14* (MYB family), *WRKY40* (WRKY family), *HSP30* (HSP family), *MPSR1* and *ZHD4* (zinc finger family), and *MADS3* and *MADS27* (MADS-box family) were significantly up-regulated more than 16-fold under low-temperature stress compared to the 0 h control (Table 1). These TFs may play an important role in responses to low-temperature stress.

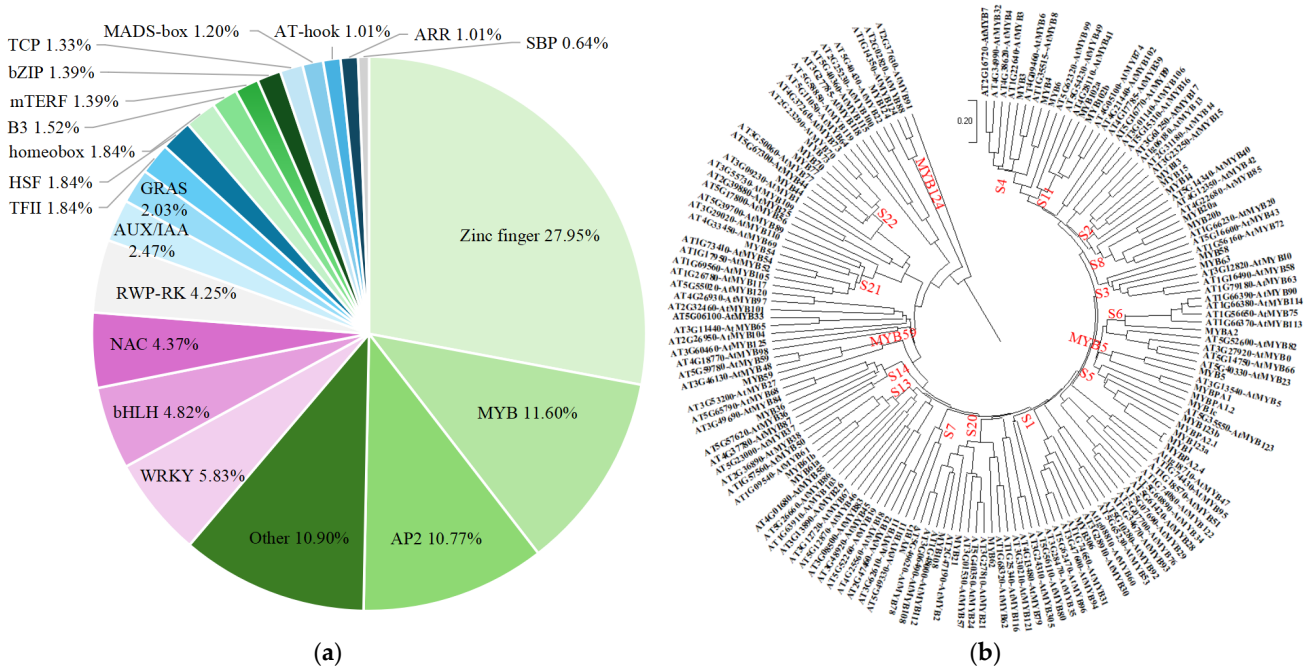


Figure 3. Differentially expressed transcription factors (TFs) during low-temperature stress in blueberry leaves identified by transcriptome deep sequencing. (**a**) Number of differentially expressed TFs in response to low-temperature stress. (**b**) The differentially expressed R2R3-MYB transcription factors from blueberry and R2R3-MYB transcription factors from *Arabidopsis thaliana* were clustered into different subgroups using MEGA X with the neighbor-joining method.

Table 1. The significantly up-regulated transcription factors during low-temperature stress.

Gene ID	Gene Family	Gene Name	Fold Change			
			L6h_vs._L0h	L12h_vs._L0h	L24h_vs._L0h	L48h_vs._L0h
VaccDscaff12-processed-gene-63.5	AP2	*CBF2*	16.68	--	--	--
VaccDscaff206-processed-gene-1.6		*ERF109*	16.34	--	--	--
VaccDscaff38-augustus-gene-244.13	MYB	*MYB14*	16.31	--	--	--
VaccDscaff20-augustus-gene-258.24	WRKY	*WRKY40*	16.87	26.62	--	--
VaccDscaff2-augustus-gene-24.22	HSF	*HSF30*	--	--	20.26	--
VaccDscaff13-processed-gene-79.20	Zinc finger	*MPSR1*	--	--	17.19	--
VaccDscaff27-augustus-gene-109.27		*ZHD4*	--	--	--	17.92
VaccDscaff22-snap-gene-30.36	MADS-box	*MADS3*	--	--	--	25.14
VaccDscaff23-augustus-gene-282.26		*MADS27*	--	--	--	30.61

After deleting redundant sequences of MYB TFs, 38 differentially expressed R2R3-MYB TFs were identified (Table S7). To predict the potential functions of these R2R3-MYB TFs, we constructed a phylogenetic tree based on the amino acid sequences of the 38 differentially expressed R2R3-MYBs from blueberry and 124 AtMYBs from *Arabidopsis* (Figure 3b). These blueberry MYB TFs belong to 14 subgroups, which were previously defined for *Arabidopsis* AtMYB TFs. In them, MYB13, MYB14, and MYB15 belonged to subgroup 2 and MYB3, MYB4, and MYB6 were clustered in subgroup 4. MYB1, MYB123a, MYB123b, MYB1c,

MYBA2.1 and MYBPA2.4 were clustered in subgroup 5, MYBA2 belonged to subgroup 6, and MYB12 belonged to subgroup 7. At the same time, some blueberry MYBs belonging to subgroup 1 (*MYB306*), subgroup 3, subgroup 8, subgroup 11, subgroup 13, subgroup 14, subgroup 20, and subgroup 22 (*MYB44*) also showed differential expression under low-temperature stress. *MYB5*, *MYBPA1*, and *MYBPA1.2* (probably homologs of *AtMYB5*), *MYB124* (probably homologs of *AtMYB124*), and *MYB59* (probably homologs of *AtMYB59*) also respond to low-temperature stress.

3.4. Metabolome Analysis in Response to Low-Temperature Stress

A total of 1202 metabolomic substances were detected from 11 superclasses in positive (746) and negative (456) ion mode in response to low-temperature stress (Figure 4a and Table S8). Among these, lipids and lipid-like molecules and phenylpropanoids and polyketides are mainly metabolomic and account for 24.38% and 23.38%, respectively. Then, organic oxygen compounds, benzenoids, organoheterocyclic compounds, and organic acids and derivatives account for about 20% (Figure 4b). The quality control (QC) samples were closely clustered together in both the positive ion mode and the negative ion mode (Figure S4). The correlations between QC samples were more than 0.989 and indicated good repeatability of the experiment (Figure S5).

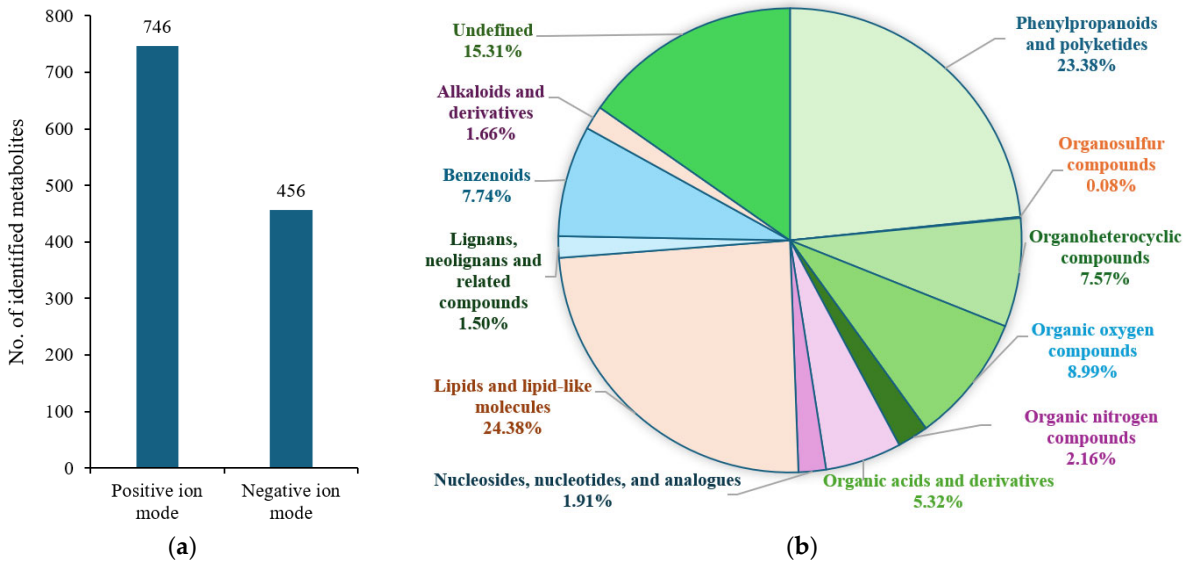


Figure 4. Metabolomic substances were identified under low-temperature stress in blueberry leaves identified by metabolomic profiling. (**a**) Number of detected metabolomic substances. (**b**) Classification of the metabolomic substances.

3.5. Differentially Accumulated Metabolites in Response to Low-Temperature Stress

To explore the effects of low-temperature stress on metabolism in blueberry leaves, we screened the DAM in positive and negative ion mode in response to low temperatures (Table S9). A total of 303 DAMs were significantly up-regulated or down-regulated under low-temperature stress. For example, 171 DAMs were identified in L6h_vs._L0h (84 up-regulated and 87 down-regulated), 53 in L12h_vs._L0h (22 up-regulated and 31 down-regulated), 174 in L24h_vs._L0h (94 up-regulated and 80 down-regulated), and 90 in L48h_vs._L0h (34 up-regulated and 56 down-regulated) (Figure 5a). The Venn diagram showed that only 8 DAMs were shared by all pairwise comparisons, 65 DAMs were identified only in L6h_vs._L0h, 15 in L12h_vs._L0h, 54 in L24h_vs._L0h, and 30 in L48h_vs._L0h, indicating that most metabolites accumulated specifically at various time points under low-temperature stress (Figure 5b). Most DAMs belonged to the phenyl-

propanoid and polyketide superclass (24.44%, 77 DAMs), and the Venn diagram showed that most phenylpropanoid and polyketide metabolites were accumulated at 6 and/or 24 h of low-temperature stress compared to the 0 h control samples (Figure 5b). We also found that flavonoid metabolites (53 DAMs) accounted for 68.83% and coumarins and derivatives (7 DAMs) accounted for 9.09% in the phenylpropanoid and polyketide superclass, indicating that flavonoid metabolites are the main DAMs under low-temperature stress (Figure 5c).

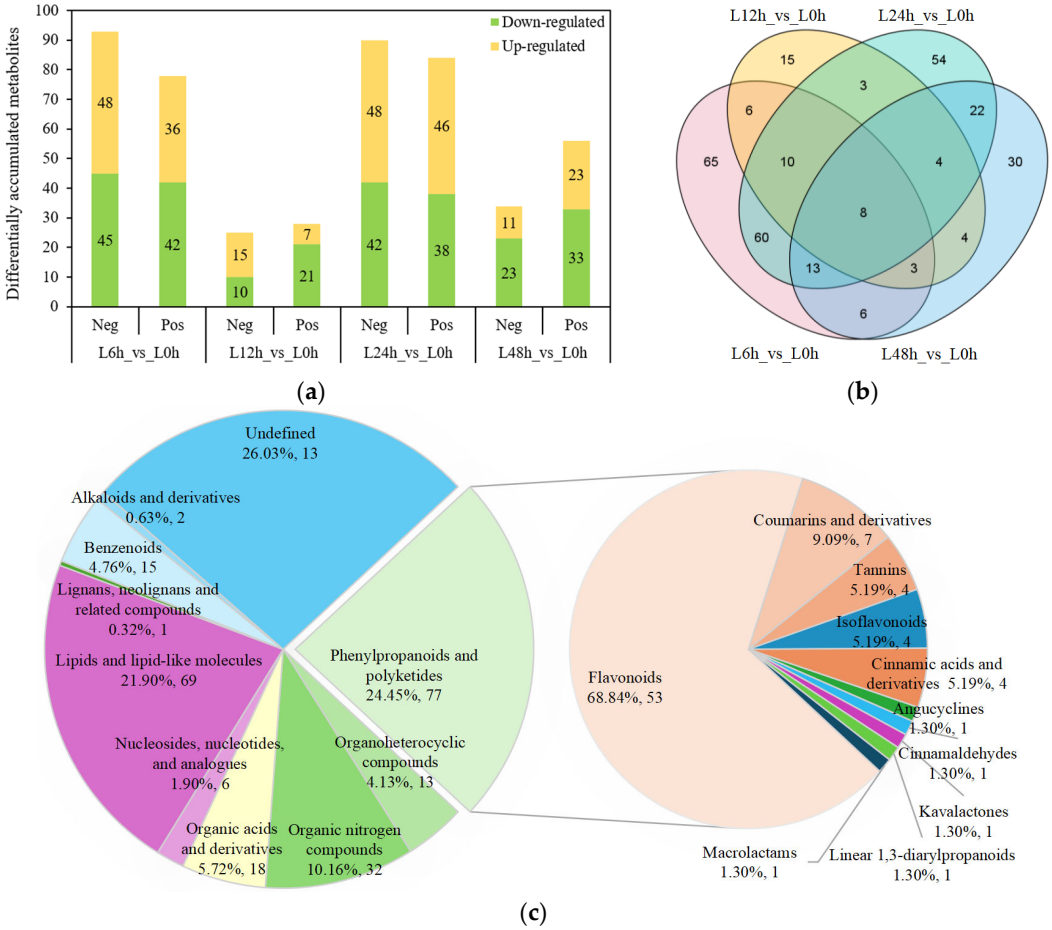


Figure 5. Differentially accumulated metabolites (DAMs) were identified during low-temperature stress in blueberry leaves identified by metabolomic profiling. (**a**) Number of DAMs in response to low-temperature stress in positive and negative ion mode; "Down-regulated" and "Up-regulated" represent down-regulated and up-regulated DAMs, respectively. (**b**) The Venn diagram shows the extent of overlap between DAMs for across pairwise comparisons. L0h, L6h, L12h, L24h, and L48h represent samples treated with low temperatures for 0, 6, 12, 24, and 48 h, respectively. (**c**) Classification of the DAMs.

3.6. Enrichment Analysis of KEGG Pathways for Differentially Accumulated Metabolites

A Venn diagram showed that all DAMs were assigned to 62 KEGG pathways; of these, 19 KEGG pathways were shared by all four pairwise comparisons (Figure S6 and Table S10). The flavonoid biosynthesis and phenylpropanoid biosynthesis pathways were significantly enriched at 6, 24, and 48 h of low-temperature stress compared to the 0 h control samples, and the flavone and flavonol biosynthesis pathway was significantly enriched in L6h_vs._L0h and L24h_vs._L0h (Figure 6). Thus, the DAMs from the phenylpropanoid biosynthesis pathway, the flavonoid biosynthesis pathway, and the flavone and flavonol biosynthesis pathway play an important role in response to low-temperature stress in blueberry leaves.

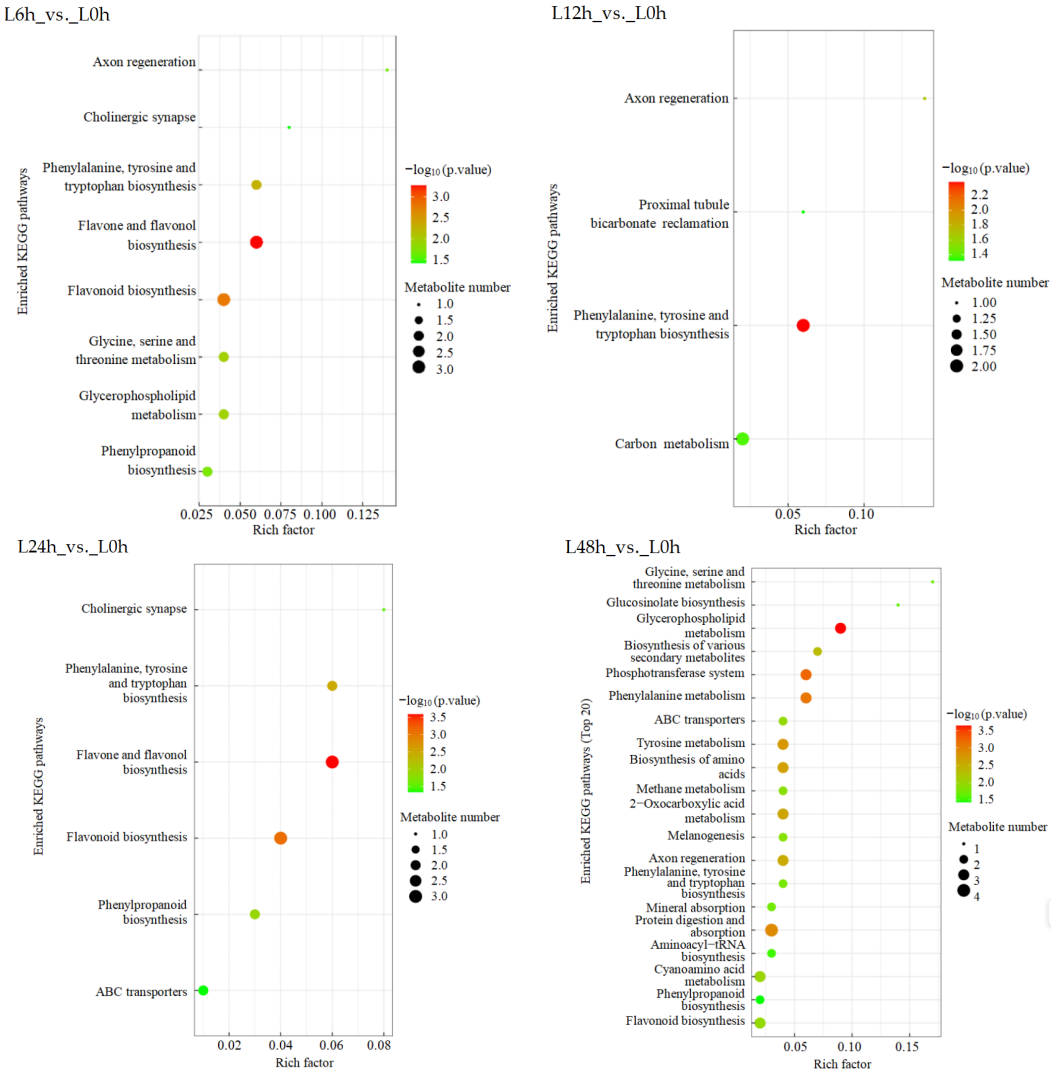
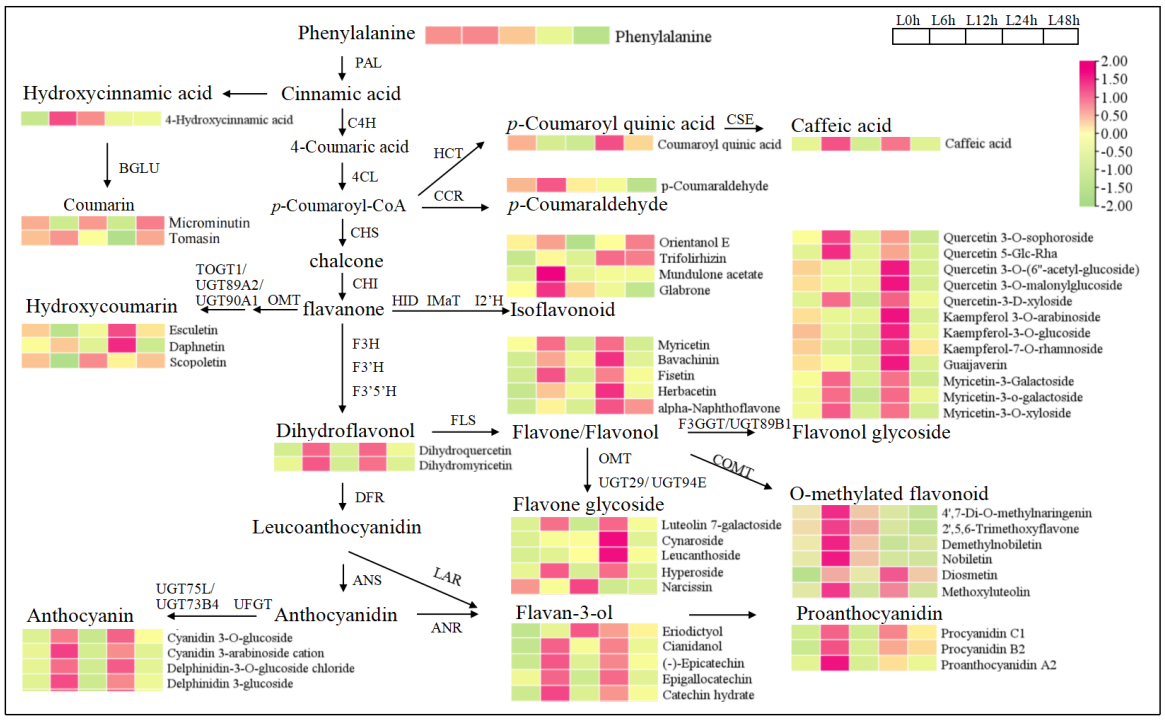


Figure 6. The top 20 KEGG enrichment analysis for differentially accumulated metabolites during low-temperature stress in blueberry leaves identified by non-targeted metabolomic profiling. L0h, L6h, L12h, L24h, and L48h represent samples treated with low temperatures for 0, 6, 12, 24, and 48 h, respectively.

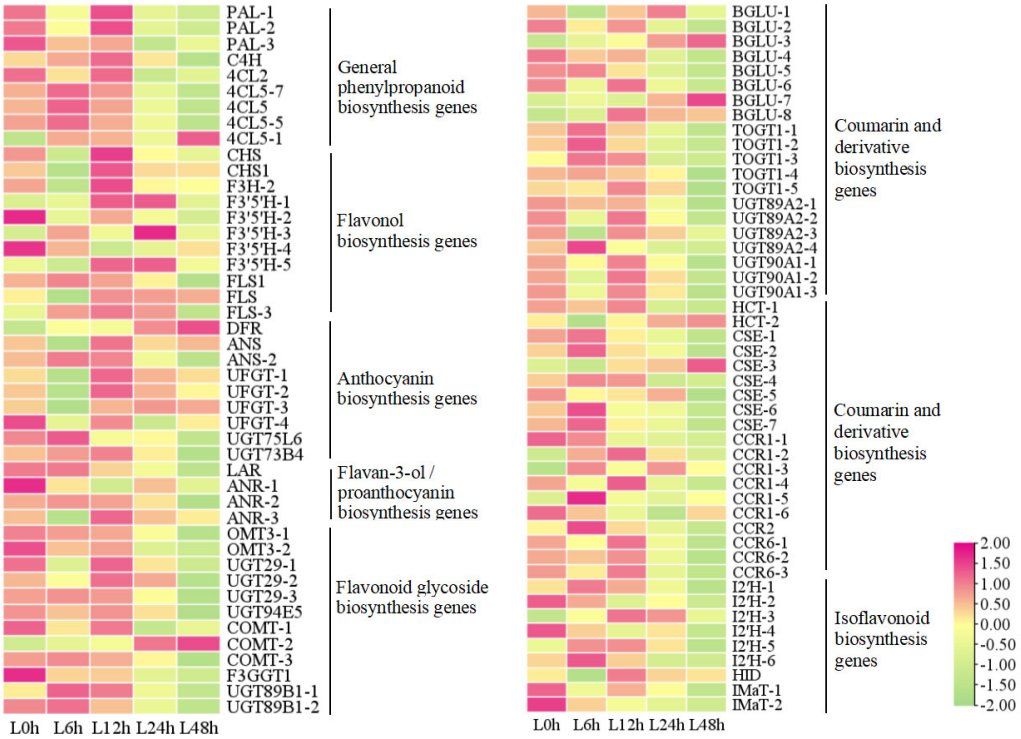
3.7. Differentially Accumulated Metabolites and Differentially Expressed Genes in Phenylpropanoid Biosynthesis KEGG Pathway Under Low-Temperature Stress

The analysis of the KEGG pathway showed that DEGs and DAMs were enriched in the phenylpropanoid biosynthesis pathway. Thus, we identified 93 DEGs encoding 30 types of enzymes involved in phenylpropanoid biosynthesis and 56 differentially accumulated phenylpropanoid metabolites including flavonoids (flavonols, flavans, anthocyanins, and proanthocyanidins), cinnamic acids and derivatives, and coumarins and derivatives in response to low-temperature stress (Table S11). Most DAMs belonging to the cinnamic acids and derivatives (caffeic acid and 4-hydroxycinnamic acid) and flavonoids (dihydroflavonols, flavonols, flavonol glycosides, flavan-3-ols, flavone glycosides, anthocyanins, and proanthocyanidins) were up-regulated at 6 or 24 h of low-temperature stress. However, most flavonoid metabolites were down-regulated at 48 h in response to low-temperature stress. Orientanol E and glabrone (isoflavonoids), microminutin and tomasin (coumarins and derivatives), and scopoletin (hydroxycoumarins) were down-regulated under low-temperature stress. In general, most differentially accumulated phenylpropanoid metabolites were up-regulated under low-temperature stress. Among them, bavachinin and

alpha-naphthoflavone (flavonols) and kaempferol-7-O-rhamnoside (flavonol glycosides) increased more than 4-fold after 24 h of low-temperature treatment and dihydromyricetin (dihydroflavonols), myricetin-3-O-xyloside and delphinidin 3-glucoside (flavonol glycosides), nobiletin (O-methylated flavonoids) and caffeic acid increased more than 3-fold after 6 h of low-temperature treatment relative to the 0 h control (Figure 7a).



(**a**)



(**b**)

Figure 7. The KEGG biosynthesis pathways of differentially accumulated phenylpropanoid metabolites and differentially expressed phenylpropanoid biosynthesis genes in blueberry leaves in response

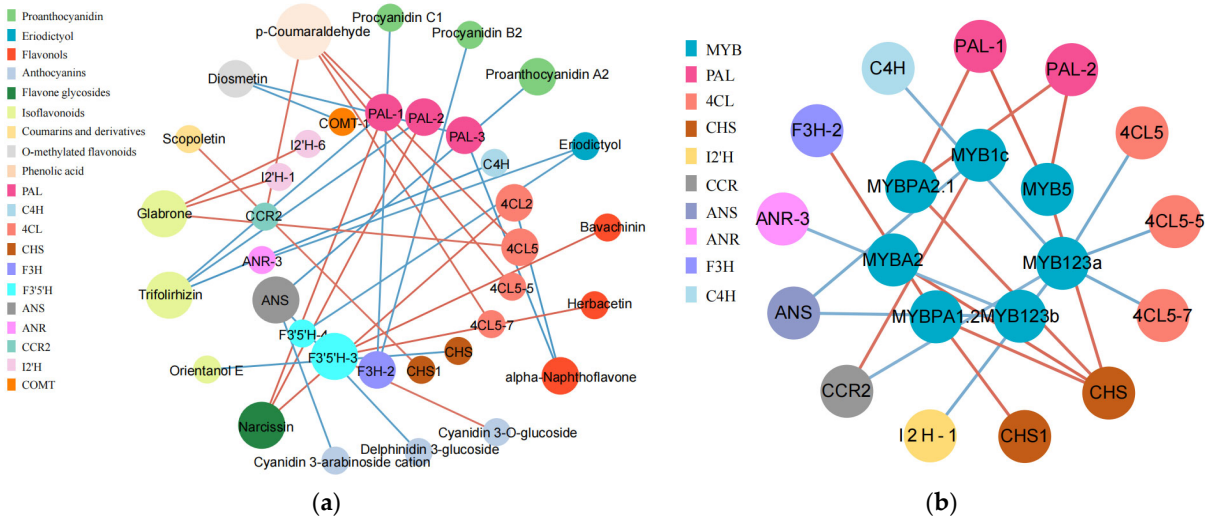
to low-temperature stress. (**a**) The phenylpropanoid KEGG biosynthesis pathways. Heatmaps show the accumulation of DAMs. Colored bars on the upper right indicate low expression (pink) or high expression (green) of differentially accumulated phenylpropanoid metabolites based on $\log_{10}$ (peak intensity). (**b**) The heatmaps represent the differentially expressed phenylpropanoid biosynthesis genes. Colored bars on the lower right indicate low expression (pink) or high expression (green) of differentially expressed phenylpropanoid biosynthesis genes based on $\log_{10}$ (FPKM). PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; HCT, shikimate O-hydroxycinnamoyltransferase; CSE, caffeoyl shikimate esterase; CCR, cinnamoyl-CoA reductase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase; UFGT, UDP-glucose flavonoid 3-O-glucosyl transferase; OMT, O-methyltransferase domain; COMT, caffeic acid 3-O-methyltransferase; HID, 2-hydroxyisoflavanone dehydratase; IMaT, isoflavone malonyltransferase; I2′H, isoflavone 2′-hydroxylase; F3GGT, flavonol-3-O-glucoside/galactoside glucosyltransferase; UGT, UDP-glycosyltransferase.

The most DEGs from phenylpropanoid biosynthesis pathway were down-regulated at 48 h of low temperature (Figure 7b). Some DEGs were up-regulated under low temperature at 6 or 12 h of low-temperature stress. Among them, *4CL5-1* (general phenylpropanoid biosynthesis gene), *F3′5′H-1*, *F3′5′H-3*, *F3′5′H-5*, and *FLS-3* (flavonol biosynthesis genes), *CSE-3*, *CCR1-2*, *CCR1-3*, and *CCR1-5* (coumarin and derivative biosynthesis genes), *I2′H-3* and *I2′H-5* (isoflavonoid biosynthesis genes), and *COMT-2*, *BGLU-3*, *BGLU-7*, and *BGLU-8* (cinnamic acid and derivative biosynthesis genes) were up-regulated, *CSE-2*, *CSE-6*, *I2′H-1*, *TOGT1-2*, and *TOGT1-3* (coumarin and derivative biosynthesis genes) and *UGT89B1-1* (a flavonoid glucoside biosynthesis gene) were up-regulated and then down-regulated, and other DEGs from the phenylpropanoid biosynthesis pathway were down-regulated in response to low-temperature stress. *4CL5-1*, *CCR1-3*, and *I2′H-5* at 6 h, *4CL5-1*, *F3′5′H-5*, *CCR1-2*, *I2′H-3*, and *I2′H-5* at 12 h, *F3′5′H-5*, *DFR*, and *CCR1-3* at 24 h, and *4CL5-1* and *DFR* at 48 h increased more than 4-fold in expression levels under low-temperature treatment compared to the 0 h control samples (Table S11). Thus, most genes involved in the phenylpropanoid biosynthesis pathway were induced during low-temperature stress.

3.8. Combined Metabolome and Transcriptome Analysis of the Phenylpropanoid Biosynthesis Pathway Under Low-Temperature Stress

The correlations between the FPKM values of differentially expressed phenylpropanoid biosynthesis genes and peak areas of differentially accumulated phenylpropanoid metabolites were calculated to investigate the functions of phenylpropanoid biosynthesis genes in response to low temperature (Table S12; Figure 8a). The proanthocyanidin metabolites procyanidin C1 and procyanidin B2 were negatively correlated with *F3H-2* and proanthocyanidin A2 was negatively correlated with *ANS* and *ANR-3* during low-temperature stress. For falvan-3-ol metabolites, only eriodictyol was negatively correlated with *F3′5′H-4*. Among the three flavonol metabolites, the levels of bavachinin and herbacetin were positively correlated with *F3′5′H-3* expression, and those of alpha-naphthoflavone was negatively correlated with *PAL-3* and *4CL2* expression. For anthocyanin metabolites, the level of cyanidin 3-O-glucoside was also positively correlated with *F3′5′H-3* expression; however, the levels of cyanidin 3-arabinoside cation and delphinidin 3-glucoside were negatively correlated with *ANS* expression. Narcissin (flavone glycoside) was positively correlated with three general phenylpropanoid biosynthesis genes (*PAL-1*, *PAL-2* and *4CL2*). The isoflavonoid metabolite orientanol E was negatively correlated with *CHS*, trifolirhizin was negatively correlated with three genes including *PAL-1*, *PAL-2*, and *C4H*, and glabrone was positively correlated with *4CL5* and isoflavone 2′-hydroxylases (*I2′H-1* and *I2′H-2*). The hydroxycoumarin metabolite scopoletin was

positively correlated with *CHS1*, and diosmetin was negatively correlated with *PAL-3* and *COMT-1*. p-coumaraldehyde was positively correlated with *4CLs* (*4CL5*, *4CL5-5*, and *4CL5-7*) and *CCR2*. These results indicate that *PAL*, *C4H*, *4CL*, *CHS*, *F3H*, *F3′5′H*, *ANS*, *ANR*, *CCR*, *I2′H*, and *COMT* gene families play important roles in regulating phenylpropanoid metabolite biosynthesis under low-temperature stress in blueberry leaves.



(**a**) (**b**)

Figure 8. The correlation analysis between the differentially accumulated phenylpropanoid metabolites and differentially expressed phenylpropanoid biosynthesis genes for blueberry leaves in response to low-temperature stress by integrative of transcriptomic and metabolomic data. (**a**) The network of correlation between differentially accumulated phenylpropanoid metabolites and differentially expressed phenylpropanoid biosynthesis pathway genes. (**b**) The network of correlation between differentially expressed phenylpropanoid biosynthesis pathway genes and differentially expressed MYB genes from group 5, group 6, and group AtMYB5.

The MYB TFs from subgroups 5, 6, and 7 and subgroup AtMYB5 regulated the phenylpropanoid metabolite accumulation by regulating the expression of phenylpropanoid biosynthesis genes [28]. To elucidate the functions of the MYBs in response to low-temperature stress in regulating phenylpropanoid metabolite biosynthesis, the Pearson correlation coefficients (*r*) were calculated between MYBs from subgroups 5, 6, and 7 and subgroup AtMYB5 and genes above phenylpropanoid biosynthesis genes (Table S13). A total of seven *MYB*s were significantly correlated with phenylpropanoid biosynthesis genes (Figure 8b). In which, *MYBA2* belonging to subgroup 6 was positively correlated with *CHS* during low-temperature stress. For subgroup 5, *MYB123a* was negatively correlated with *C4H*, *4CL5-7*, *4CL5*, *4CL5-5*, *CCR2*, and *I2′H-1* genes, *MYB123b* was negatively correlated with *ANS* and *ANR-3*, *MYB1c* was positively correlated with *CCR2* and negatively correlated with *ANS*, and *MYBPA2.1* was positively correlated with *PAL-1*, *PAL-2*, and *CHS*. *MYBPA1.2* and *MYB5* were homologues of AtMYB5; *MYBPA1.2* was positively correlated with *CHS*, *CHS1*, and *F3H-2*, and *MYB5* was positively correlated with *PAL-1*, *PAL-2*, and *CHS*.

4. Discussion

4.1. The DEGs and DAMs in Response to Low-Temperature Stress in Blueberry Leaves

The response of plants to low-temperature stress is accompanied by changes in the expression patterns of many genes [29]. Here, we found that 16,388 DEGs were up-regulated or down-regulated during low-temperature stress and were assigned to 271 KEGG pathways. In *Arabidopsis*, *CBF1*, *CBF2*, and *CBF3* function redundantly in regulating a large

number of *COR* genes and are critical in freezing tolerance [30–32]. In blueberry, overexpression of the *CBF* gene exhibited enhanced freezing tolerance [33,34]. Here, the *CBF2* gene (the same gene with *CBF*) was up-regulated more than 16-fold during low-temperature stress and may play an essential role in increasing low-temperature stress tolerance. At the same time, we screened nine *COR* genes, among which seven genes were up-regulated and two genes were down-regulated. Among them, *COR1*, *COR2*, and *COR413PM1* were up-regulated more than 3-fold during low-temperature stress. The expression of *COR* genes has been shown to be critical in low-temperature-tolerance in plants and is regulated by *CBF*s [35,36]. In *Arabidopsis*, ICE1 (bHLH TF) can bind to the *CBF3* promoter and is important for the expression of *CBF3* during cold acclimation. The overexpression of *ICE1* enhanced the expression of *CBF3*, *CBF2*, and *COR* genes during cold acclimation and increased the freezing tolerance of the transgenic *Arabidopsis* [37]. Here, two *ICE1* genes were induced under low-temperature stress. Therefore, the *ICE1*, *ABF*, and *COR* genes, especially *ABF2*, play an important role in improving blueberry tolerance to low-temperature stress. We also found that the DEGs from the phenylpropanoid biosynthesis pathway was significantly enriched; the expression levels of *4CL5-1*, *DFR*, *F3′5′H-5*, *CCR1-2*, *CCR1-3*, *I2′H-3*, and *I2′H-5* genes increased more than 4-fold during low temperatures compared to 0 h control samples. These genes may contribute to plant tolerance to low-temperature stress in blueberry leaves [38,39].

In plants, TFs are involved in the regulation of the low-temperature stress response [40,41]. Here, 1578 TFs were differentially expressed under low temperature; these TFs mainly come from zinc finger, MYB, AP2, WRKY, bHLH, and NAC families. Among them, HD-Zip TFs (zinc finger family) HaHB1, and AtHB13 confer cold tolerance [42]. WRKY Ifs, as molecular switches, regulate low-temperature stresses in plants; for example, *CsWRKY2* (*Camellia sinensis*) is involved in cold stress responses, and *KoWRKY40* (*Kandelia obovata*) and *MbWRKY40* (*Malus baccata*) enhance cold tolerance in transgenic *Arabidopsis* [11,43,44]. The AP2/ERF family in plants is involved in plant multiple environmental stimuli [12]. The most famous members of the AP2/ERF family involved in cold stress are *DREB*s, also known as *CBF*s. As pioneers of regulatory networks in response to cold stress, *CBF*s can increase the expression of cold-regulated genes and improve cold tolerance in many plants [45,46]. The members of HSP and MADS-box families are also involved in abiotic stress [47,48]. In this study, *CBF2* and *ERF109*, *WRKY40*, *HSP30*, *MPSR1*, *ZHD4*, *MADS3*, and *MADS27* were significantly up-regulated more than 16-fold during low-temperature stress. Therefore, we predict that these TFs may be related to low-temperature tolerance in the blueberry leaves.

R2R3-MYB family TFs have been found to mediate low-temperature stress responses in plants. Apple *MdMYB88* and *MdMYB124* regulate frost tolerance and cold response gene expression [17]. In *Arabidopsis*, *AtMYB14* and *AtMYB15* participate in freezing tolerance, the *AtMYB44* gene is activated under low-temperature stress and the overexpression of *AtMYB44* enhances abiotic stress tolerance, and *AtMYB96* can be induced by cold stress and activates freezing tolerance [14,15,49]. In this study, 38 blueberry R2R3-MYB TFs were induced by low-temperature stress. Of these, *MYB13*, *MYB14* (probably homologous to *AtMYB14*) and *MYB15* (probably homologous to *AtMYB14*) from subgroup 2 were induced by low-temperature stress. We also found that *MYB44* (probably homologous to *AtMYB44*), *MYB124* (probably homologous to *MdMYB124*), and *MYB306* from group 1 (the same group with *AtMYB96*) were also activated under low-temperature stress. Thus, *MYB13*, *MYB14*, *MYB15*, *MYB44*, *MYB124*, and *MYB306* play crucial roles in low-temperature stress for blueberry leaves.

Low temperatures are the main factor affecting the growth of blueberry plants in northern China [50]. Plants adapt to environmental changes by adjusting metabolite

accumulation under stress [51]. Most DAMs belong to the phenylpropanoid and polyketide superclass. Low temperatures promote the rapid accumulation of these metabolites. Among them, dihydromyricetin, myricetin-3-O-xyloside, delphinidin 3-glucoside, nobiletin, and caffeic acid increased more than 3-fold and bavachinin, alpha-naphthoflavone, and kaempferol-7-O-rhamnoside increased more than 4-fold during low-temperature stress. These metabolites have potential drug development value in many fields, such as anti-inflammatory and antitumor treatments, nervous system diseases, and diabetes [52]. These results indicate that blueberry may mitigate damage from low-temperature stress by promoting the accumulation of these phenylpropanoid metabolites.

4.2. The Regulatory Network of Low-Temperature Stress-Induced Accumulation of Phenylpropanoid Metabolites in Blueberry Leaves

The *PAL*, *C4H*, and *4CL*, as the general phenylpropanoid biosynthesis pathway genes, regulated phenylpropanoid metabolite biosynthesis under low-temperature stress [53,54]. Here, we found that *PAL-1* and *PAL-2* were positively correlated with narcissin and negatively correlated with trifolirhizin and *PAL-3* was negatively correlated with alpha-naphthoflavone and diosmetin. *C4H* was negatively correlated with trifolirhizin. *4CL2* was positively correlated with narcissin and was negatively correlated with alpha-Naphthoflavone. *4CL5* was positively correlated with glabrone and p-coumaraldehyde and *4CL5-5* and *4CL5-7* were also positively correlated with p-coumaraldehyde. Thus, it is likely that *PAL-1* and *PAL-2* are the key genes in narcissin biosynthesis, and *PAL-3* is one of the key genes in alpha-naphthoflavone and diosmetin biosynthesis under low-temperature stress. *C4H* might be the key gene in trifolirhizin biosynthesis under low-temperature stress. 4CL family members may play key roles in alpha-naphthoflavone, narcissin, glabrone, and p-coumaraldehyde biosynthesis during low-temperature stress.

The *CHS* gene regulates isoflavonoid, flavonoid, and hydroxycoumarin biosynthesis [55,56]. In this study, *CHS* was negatively correlated with orientanol E and *CHS1* was positively correlated with scopoletin during low-temperature stress. Orientanol E belongs to the isoflavonoid class and may be regulated by *CHS* in blueberry leaves under low-temperature stress. The *F3H* and *F3′5′H* were "early" flavonoid biosynthesis genes. *ScF3′5′H* from *Senecio cruentus* promotes anthocyanin biosynthesis [57]. Here, *F3H-2* was negatively correlated with procyanidin C1 and procyanidin B2 and *F3′5′H-3* was positively correlated with bavachinin, herbacetin, and cyanidin 3-O-glucoside. Thus, *F3H-2* may be one of the key genes in proanthocyanidin biosynthesis and *F3′5′H-3* may be one of the key genes in flavonol and anthocyanin biosynthesis under low-temperature stress, respectively.

The *ANS* from *Vaccinium* spp. is the key factor for modulating anthocyanin production [58]. In mango fruit peel, *ANS* is also crucial for anthocyanin and proanthocyanidin biosynthesis [59]. Here, *ANS* was significantly correlated with proanthocyanidin A2, cyanidin 3-arabinoside cation and delphinidin 3-glucoside, indicating that *ANS* plays an important role in anthocyanin and proanthocyanidin biosynthesis. ANR is a pivotal enzyme that diverts the flavonoid pathway from anthocyanins to proanthocyanidin synthesis [60]. In this study, *ANR-3* was significantly correlated with proanthocyanidin A2. Therefore, *ANR-3* may be the key gene for the biosynthesis of proanthocyanidin A2 in blueberry leaves under low-temperature stress. In plants, *p*-coumaraldehyde is a direct precursor to H-type monolignol biosynthesis, which is synthesized by the members of the CCR family [61]. This study showed that *CCR2* was significantly correlated with p-coumaraldehyde, indicating *CCR2* may be the key gene in p-coumaraldehyde biosynthesis in blueberry leaves during low-temperature stress. *I2′H* is an isoflavonoid biosynthesis pathway gene, and *COMT* is an O-methylated flavonoid biosynthesis pathway gene [62,63]. Here, *I2′H-1* and *I2′H-6* were significantly correlated with glabrone, and *COMT-1* was significantly correlated with diosmetin during the low-temperature stress. Thus, *I2′H-1* and *I2′H-6* and *COMT-1* play

important roles in regulating glabrone and diosmetin biosynthesis in blueberry leaves during low-temperature stress, respectively.

In *Arabidopsis*, the R2R3-MYB family members are divided into 25 subgroups [28,64], in which subgroup 5 (AtMYB123), subgroup 6 (AtMYB75/90/113/114), and subgroup 7 (AtMYB11/12/111) positively regulate proanthocyanidin, anthocyanin, or flavonol biosynthesis [65–68]. The gene *VvMYB5b* from grape is homologous with *AtMYB5*, which is associated with anthocyanin and proanthocyanidin biosynthesis [69]. *MtMYB5* (*Medicago truncatula*) and *DkMYB4* (*Diospyros kaki*), the homolog of *AtMYB5*, have also been demonstrated to regulate proanthocyanidin accumulation [70,71]. Here, *MYB1*, *MYB123a*, *MYB123b*, *MYB1c*, *MYBA2.1*, and *MYBPA2.4* (subgroup 5), *MYBA2* (subgroup 6), *MYB12* (subgroup 7), and *MYB5*, *MYBPA1*, and *MYBPA1.2* (probably homologs of *AtMYB5*) were induced by low-temperature stress in blueberry leaves. For *Erigeron breviscapus*, *EbMYBP1* participated in flavonoid biosynthesis by activating the transcription of flavonoid-associated genes (*FLS*, *F3H*, and *CHS*) [72]. In apple (*Malus sieversii* f. *niedzwetzkyana*), *MYB12* could bind to the promoters of *UFGT*, *ANR*, and *LAR*, and *MYB22* could bind to the promoters of *UFGT* and *FLS*, promoting the accumulation of proanthocyanidin and flavonol in apple callus [73]. In this study, the blueberry MYB TFs belonging to subgroup 6 (MYBA2), subgroup 5 (*MYB123a*, *MYB123b*, *MYB1c*, and *MYBPA2.1*) and subgroup AtMYB5 (*MYB5* and *MYBPA1.2*) were significantly correlated with phenylpropanoid biosynthesis genes, indicating that these MYB TFs may be involved in low-temperature stress-induced phenylpropanoid biosynthesis by regulating the expression of phenylpropanoid biosynthetic genes (Figure 9).

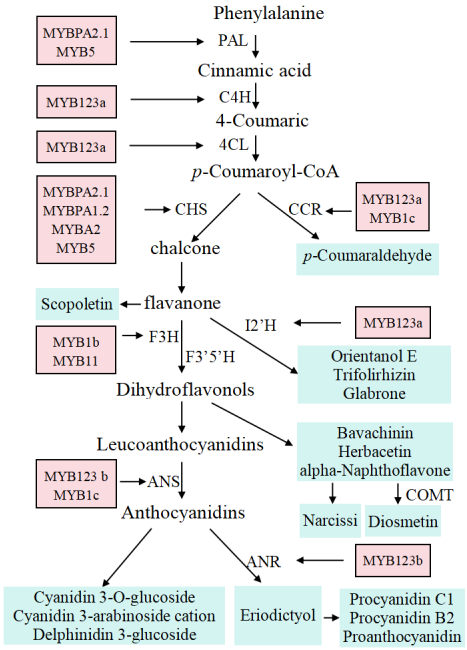


Figure 9. A regulatory network identified in this study via the integration of transcriptomic and metabolomic data in low-temperature stress-induced accumulation of phenylpropanoid metabolites in blueberry leaves.

5. Conclusions

In this paper, we analyzed the transcriptional and metabolic responses of blueberry leaves to low-temperature stress via RNA-seq and UHPLC-MS/MS, respectively. Transcriptomic analysis indicated that low-temperature stress induced expression of *ICE-CBF-COR* regulatory pathway genes, phenylpropanoid biosynthesis genes, and TFs and significantly up-regulated the expression of *CBF2*, *ERF109*, *MYB14*, *WRKY40*, *HSP30*, *MPSR1 ZHD4*,

MADS3, and *MADS27* genes. Metabolomics analysis showed that low-temperature stress induced the accumulation of phenylpropanoid metabolites and significantly promoted the accumulation of dihydromyricetin, myricetin-3-O-xyloside, delphinidin 3-glucoside, kaempferol-7-O-rhamnoside, nobiletin, caffeic acid bavachinin, and alpha-naphthoflavone. Integrative transcriptomic and metabolomic analysis indicated that *MYBA2*, *MYB123a*, *MYB123b*, *MYB1c*, *MYBPA2.1*, *MYB5*, and *MYBPA1.2* regulated low-temperature-induced accumulation of phenylpropanoid metabolites in blueberry leaves by regulating the expression of *PAL*, *C4H*, *4CL*, *CHS*, *F3H*, *ANS*, *CCR*, *I2′H*, and *ANR* genes. In this study, we screened the genes and metabolites that respond to low-temperature stress in blueberry leaves. Whether these genes and metabolites improve the low-temperature tolerance of blueberry leaves requires further functional verification. At the same time, it is not clear whether low-temperature stress induces the expression of these genes and accumulation of these metabolites in fruits and roots. Our findings will provide a basis for future studies aimed at cultivating low-temperature-resistant blueberry varieties.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae11121495/s1.

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