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Rootstock-Mediated Transcriptional Changes Associated with Cold Tolerance in *Prunus mume* Leaves

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Abstract: Japanese apricot (*Prunus mume*) is remarkably valuable for its high ornamental and economic importance due to its distinctive features. Low temperature is a serious environmental constraint for this species, restricting its cultivation and dispersal in the north of China. To address this issue, breeding requires an understanding of the molecular mechanisms underlying responses to cold stress. We examined the leaf physiological and transcriptome profile by RNA sequencing in 'Bungo' scion cultivar grafted onto *Prunus mume* (cold-sensitive) and *Prunus armeniaca* (cold-tolerant) rootstocks at 4 °C for 0, 6, and 24 h. Our results revealed that the increased MDA concentration in the leaves of *P. mume* cultivar (cold-sensitive) suggests that cold stress might cause oxidative damage and increased sensitivity. Moreover, the cold-tolerant cultivar (*P. armeniaca*) considerably enhances the enzyme activities (i.e., SOD, POD, and CAT), as well as osmo-protectants (soluble sugars and proline) compared with sensitive cultivar, which helps plants to withstand oxidative damage caused by cold stress. Additionally, differentially expressed genes were shown to be enriched in plant hormone signal transduction, ribosome, MAPK signaling, and circadian rhythm pathway. After 24 h of cold stress, genes related to *PYL4*, *histidine kinase 1*, *SAUR36*, *bHLH130*, *bHLH123*, *TIFY 6B-like*, *WRKY 40*, *WRKY 57*, and *60S acidic ribosomal protein P1* were differentially expressed, implying that these DEGs involved in multiple pathways are involved in cold tolerance in Japanese apricot. This study improved our current understanding of the mechanism of cold tolerance in Japanese apricot, and the findings could be utilized for other related fruit species.

Keywords: Japanese apricot; cold stress; physiology; biochemical analysis; transcriptome analysis; differential expressed genes

Citation: Hayat, F.; Ma, C.; Iqbal, S.; Huang, X.; Omondi, O.K.; Ni, Z.; Shi, T.; Tariq, R.; Khan, U.; Gao, Z. Rootstock-Mediated Transcriptional Changes Associated with Cold Tolerance in *Prunus mume* Leaves. *Horticulturae* **2021**, *7*, 572. https://doi.org/10.3390/horticulturae7120572

Academic Editors: Jinzhi Zhang, Pingxian Zhang and Changfei Guan

Received: 10 November 2021
Accepted: 7 December 2021
Published: 13 December 2021

1. Introduction

Japanese apricot *(Prunus mume* Sieb. et Zucc) is a popular ornamental plant in China; however, its utilization in northern China is severely limited due to the low temperatures [1,2]. Low temperature is an important abiotic stress factor that negatively impacts plant growth and development and ultimately influences the geographical distribution of plants [3–5]. Therefore, understanding the molecular responses of *P. mume* to cold stress is important for breeding cold-tolerant cultivars.

Plants have developed various adaptive mechanisms to deal with cold stress and protect themselves from cold injury [5–7]. Numerous studies exhibited that cold stress largely influences plant biochemical and physiological processes, such as rapid alterations

in malondialdehyde (MDA) content, increased levels of ROS-scavenging enzymes, an upregulation of antioxidant enzymes, the inhibition of photosynthesis and alteration in hormone levels, and these have been linked with modulation of gene transcription [8–10]. RNA sequencing has discovered many cold-regulated genes in several crops, including *Arabidopsis* [11], maize [12], rice [13], peach [14], and loquat [10].

It has been found that cold tolerance and acclimation are linked with various biological activities and complex regulatory networks [15,16]. Cold stress alters plant gene expression globally. Several transcription factors are associated with the gene regulatory networks of cold stress resistance. Previous studies have revealed that photosynthesis is impaired by cold stress due to a decrease in photosystem II activity. The decline in photosynthesis rate differs between genotypes with varying cold tolerance [17]. The signaling components include plant hormones and mitogen-activated protein kinase (MAPK) signaling, protein phosphatases, and calcium-binding proteins [18]. It is essential to understand the signaling pathways involving plant hormones and MAPK signaling. These signaling pathways are linked with cold-responsive components, which are regulated by calcium and hormonal levels [19]. Cold tolerance is the direct consequence of the timing and combination of different signaling pathways. The calcium-binding proteins detect the increase in cytosolic calcium when plants are exposed to cold stress. These calcium-binding proteins regulate key stress-responsive genes and TFs, resulting in cold stress tolerance [20].

Dehydration-responsive element-binding protein (*DREB*) is another gene family that is largely studied for cold tolerance in plants [21,22]. Furthermore, more than 1000 Arabidopsis genes have been discovered that respond to cold temperatures [23]. These cold-responsive genes include *COR* and *CRT/DRE* or *CBFs* genes [24,25].

Phytohormones, such as gibberellin (GA) and abscisic acid (ABA), have long been thought to play a role in cold response [26,27]. Many studies have confirmed that cold stress increases endogenous ABA concentrations, and exogeneous ABA treatment improves plant cold tolerance [28,29]. Another study indicates that cold stress affects plant growth and development that are closely connected with auxin intracellular IAA gradient [30]. Another essential hormone, brassinosteroids (BRs), responds to abiotic stresses, particularly cold stress [31]. The activation of COLD-RESPONSIVE (*COR*) genes by BRs has been found to improve cold tolerance [32]. The complex interactions and crosstalk among plant hormones in response to cold stress affect several physiological processes. Furthermore, sugar metabolism in plants has always been associated with hormone signaling that helps to stimulate growth, development, or even the plant's ability to respond against stress [33–35]. Variability in the levels of stress-responsive phytohormones is critical for regulating stress signaling [36]. However, the role of hormones in abiotic stress, particularly cold stress tolerance, in *P. mume* remains largely unknown.

Limited research has exposed the relationship between hormones and other biological processes. While, recent advances in the study of omics technology have revealed key biological processes influenced by plant hormones [37,38]. RNA-seq (RNA-sequencing) is a transcriptome profiling technique that employs deep-sequencing technologies [39,40]. In the present study, we employed a comparative RNA-Seq based transcriptome analysis to investigate the transcriptional changes related to cold tolerance in Japanese apricot. This research illustrates a theoretical foundation for the cold tolerance mechanism and has industrial significance for enhancing the production and quality of *P. mume*.

2. Materials and Methods

2.1. Plant Materials and Cultivation Conditions

This research was conducted at the National Field Gene bank for Japanese apricot, Nanjing Agricultural University, China (Long. 118°46′ E, lat. 32°03′ N) during March–June 2020. Japanese apricot scion cultivar 'Bungo' [*Prunus mume* Sieb. et Zucc)] and two *Prunus* rootstocks (propagation from cuttings) including '*P. mume*' [PM: cold-sensitive] and '*P. armeniaca*' [PA: cold-tolerant] were used as a test material to identify the mechanism of tolerance to cold stress. Two different scion/rootstock combinations were used as

(1) 'Bungo' grafted onto *P. mume* rootstock (Bungo/PM) and (2) 'Bungo' grafted onto '*P. armeniaca*' rootstock (Bungo/PA). The annual branches of *P. mume* 'Bungo' were collected from 7 years old mother plant and splice-grafted onto '*P. mume*' and '*P. armeniaca*'. Grafted plants were grown in plastic pots (30 cm diameter) with a mixture of nursery substrate, garden soil, and sand (3:2:1). The plants were drip irrigated, but no fertilizer was applied during the study period. The grafted plantlets were cultivated in a greenhouse with a photoperiod of 16/8 h (day/night) at 25 °C and a constant relative humidity of 70% for 2 months. For cold treatment, grafted plants (60 days: two months after grafting) were transferred to a growth chamber at (4 °C) for 6 and 24 h. The plants in the control group were kept under a normal temperature of 25 °C (control: CK) and named 0 h. The leaf samples were taken at various time points (0, 6, and 24 h) and thus employed as a source for Illumina sequencing. The samples were kept at −80 °C until further examination.

2.2. Determination of MDA, Soluble Sugars, and Proline Contents

The levels of malondialdehyde (MDA) were determined by the thiobarbituric acid method [41]. To reduce the possibility of interference from different substances along with sugars, the MDA content was determined using absorbance at 450, 532, and 600 nm as described [42,43]. The proline content was measured using the method described by Subramanyam [44], and a spectrophotometer was used to measure light absorption at 520 nm. The procedure adopted by Zhang [45] was used to measure soluble sugar contents with little modification using absorbance at 620 nm.

2.3. Determination of Different Antioxidant Enzymatic Activities

Leaf tissues (0.5 g) were grounded to a fine powder and homogenized in 5 mL of extraction buffer (phosphate buffer, pH 7.5, containing 0.1 mM EDTA, and 4% polyvinylpolypyrrolidone). The homogenate was then centrifuged for 20 min at 12,000× *g*, and the supernatant was used for enzyme analysis [46]. The antioxidant enzymes activities, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD), were measured using the methods followed by Wang [47].

2.4. RNA Extraction and Library Preparation

Total RNA was extracted from leaf tissues using Foregene Nucleic Acid Extraction Kit (Shenzhen, China), following the manufacturer's standard protocol. An Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used to determine the integrity of the RNA, and the quality was determined by gel electrophoresis. Subsequently, the RNA was treated with oligo (dT) and then combined with fragment buffer to synthesize the first-strand cDNA. Then, the fragment was purified using 1-A nucleotide addition and EB end repair. Subsequently, the ligation product was amplified by PCR technique, which was then sequenced using Illumina HiSeq 4000 (BGI, Beijing, China).

2.5. Analysis of Transcriptome Sequencing

After sequencing, the raw reads (FASTQ) were cleaned using the SOAPnuke (v. 1.4.0) software to eliminate low-quality reads, adaptor sequences, and unknown bases. To assess the quality of the clean reads, the Q20, Q30 and GC content were computed. The clean reads were mapped to *P. mume* reference genome available at (https://www.ncbi.nlm.nih.gov/genome/?term=prunus%20mume) (accessed on 6 December 2021) using HISAT (v. 2.1.0). The gene expression level was determined using RSEM (v. 1.2.8) [48].

2.6. Screening of Differentially Expressed Genes (DEGs)

The expression levels of each sequence library were analyzed using the FPKM method [49]. TBtools was used to perform hierarchical clustering on the dataset. The DESeq R program was used to identify the most important cold-responsive genes [40], default criteria with an absolute $\log_2$ fold change of $\geq \pm 1$ (upregulation) and ≤ -1 (downregulation) with adjusted

p-values less than 0.001. In order to adjust for *p*-value errors, the false discovery rate (FDR) method approach was used [50].

2.7. Gene Ontology and KEGG Pathway Analysis

GO enrichment analysis was carried out to classify the genes into three categories: molecular functions, cellular components, and biological processes using phyper package in R software [51]. GO analysis was performed with FDR $\leq$ 0.05, deliberated as considerably enriched by DEGs. Additionally, KEGG pathway analysis was used to identify the enrichment pathways.

2.8. Quantitative Real-Time PCR (qRT-PCR) for Expression Validation

Genes were randomly chosen to validate the accuracy of RNA-Seq data through RT-qPCR. The Beacon Designer-8 software was used to design primers for the selected genes. The RNA extraction and quality testing were carried out as previously reported, and qRT-PCR was done in accordance with the procedure followed by [52,53]. To calculate the relative gene expression, RPII was employed as an internal control, and the standard comparative method was used [54].

2.9. Statistical Analysis

The current study was conducted in a completely randomized design (CRD) with two factors (sampling points and rootstocks) and three replications. Data were analyzed according to analysis of variance (ANOVA) using Statistics 8.1 software package. Means were compared by using the Least Significant Difference (LSD) test. Means sharing any same letters are not statistically different, and vice versa.

3. Results

3.1. Physiochemical Characteristics of Grafted Seedlings under Cold Stress

In the present study, cold stress substantially increased MDA levels in both grafted combinations; however, the maximum accumulation of MDA under cold treatment was observed in sensitive (Bungo/PM) plants at 6 and 24 h compared to tolerant (Bungo/PM) plants (Figure 1A). Soluble sugar and proline levels increased considerably at 6 and 24 h under cold stress in the leaves of Bungo/PA grafted plants compared to Bungo/PM grafted plants (Figure 1B,F). Cold stress noticeably enhanced the antioxidant enzyme activity compared with normal temperature (control). The SOD, POD, and CAT content increased sharply at 6 and 24 h compared with the control (0 h) treatment under cold stress (Figure 1C–E). In addition, compared to Bungo/PM plants, Bungo/PA plants had higher antioxidant enzyme activities. These findings revealed a better cold tolerance response in plants grafted on '*P. armeniaca*' compared to that grafted on '*P. mume*', and makes them a suitable genetic material for further transcriptional studies.

3.2. Overview of RNA Sequencing

The Japanese apricot scion cultivar [*P. mume*, 'Bungo'] grafted on *P. mume* and *P. armeniaca* rootstocks were employed as experimental materials to examine transcriptional responses in the leaves of Japanese apricot under cold stress. cDNA libraries were made using the total RNA extracted from leaf samples. In the present study, 45.86 million raw reads were acquired and filtered. Approximately 42.58 million clean reads were acquired and mapped to the Japanese apricot (*P. mume*) reference genome. We obtained a total of 88.49% mapped reads from each sample, with 56.62% uniquely mapped (Table 1; File S1). This mapped data is shown to be reliable for DEGs analysis.

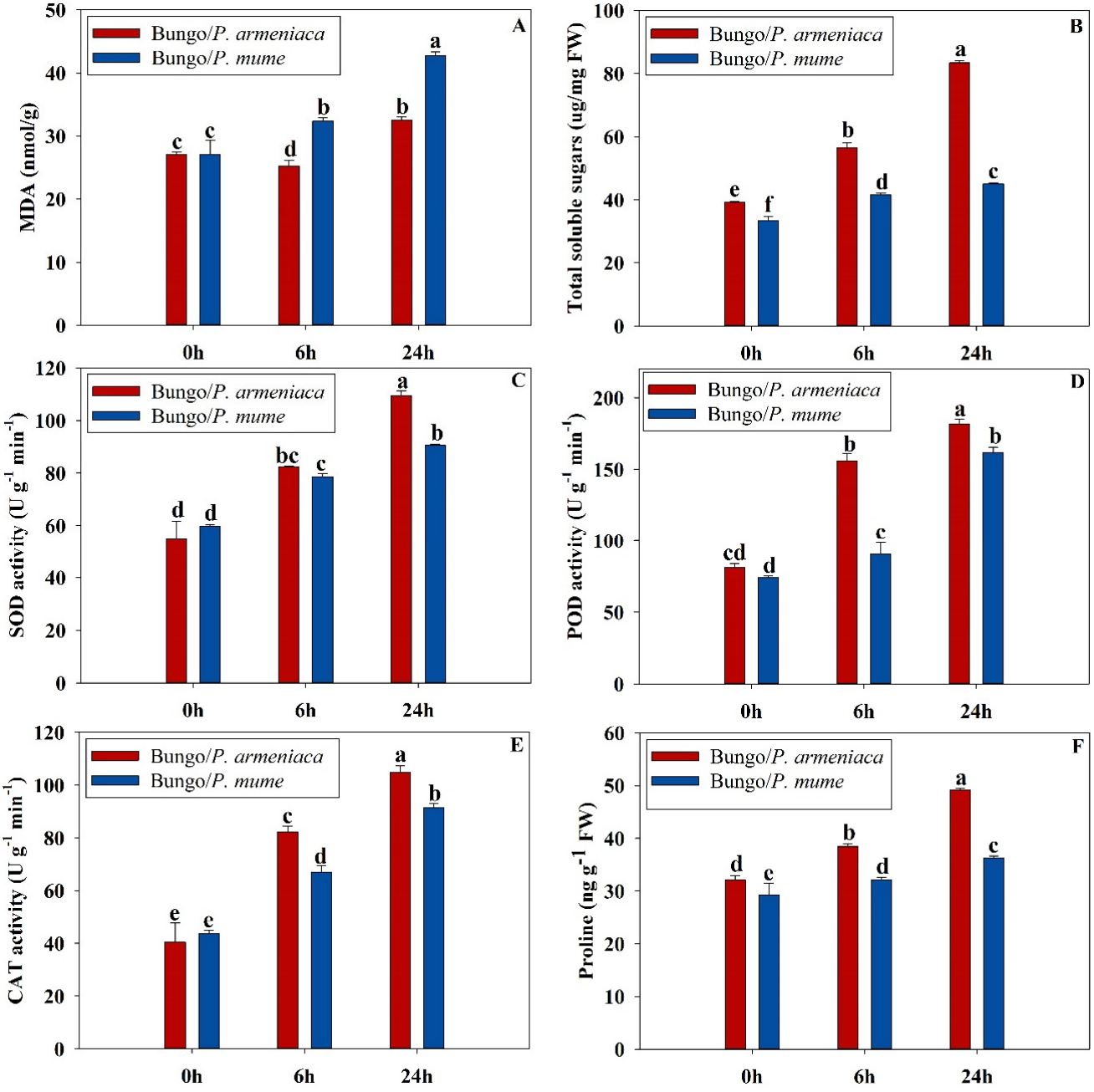

Figure 1. MDA (**A**), soluble sugars contents (**B**), SOD (**C**), POD (**D**), CAT (**E**), and proline (**F**) in cold-sensitive (Bungo/*P. mume*) and cold-tolerant (Bungo/*P. armeniaca*) grafted plants at 0, 6, and 24 h under cold stress. Data is represented as means ± standard deviation. Means sharing any same letters are not statistically different, and vice versa.

3.3. Differentially Expressed Genes (DEGs) Analysis

In the present study, transcriptome dynamics identified putative candidate genes related to the cold tolerance mechanism in *P. mume*. Three different comparisons were generated from leaves of 'Bungo' grafted on *P. mume* and *P armeniaca* rootstocks at different sampling time points. Pairwise comparison analysis was employed and compared the transcript levels of key unigenes among different samples. In comparison Bungo/PM0 vs. Bungo/PA0, identified 54 unigenes (35 upregulated and 19 downregulated), Bungo/PM6 vs. Bungo/PA6 identified 8 unigenes (8 upregulated and 0 downregulated), Bungo/PM24 vs. Bungo/PA24 identified 248 unigenes (162 upregulated and 86 downregulated) (Figure 2A). The volcano plot shows the entire distribution of differentially

expressed genes among two groups (Figure 2B–D). Expression pattern values of all the genes in these comparisons are listed in File S2.

Table 1. An overview of sequencing assembly in scions of 'Bungo' Japanese apricot scion cultivar grafted onto *P. mume* (PM) and *P. armeniaca* (PA) under cold stress.

Sample	Total Raw Reads (M)	Total Clean Reads (M)	Clean Reads Q20 (%)	Clean Reads Q30 (%)	Clean Reads Ratio (%)	Total Mapping (%)	Uniquely Mapping (%)
PA0h	46.16	42.65	97.75	93.80	92.41	88.03	56.19
PA6h	45.57	42.47	97.63	93.48	93.18	87.62	54.40
PA24h	46.16	42.68	97.78	93.87	92.46	87.11	55.28
PM0h	46.16	42.71	97.77	93.85	92.54	89.26	58.07
PM6h	45.57	42.54	97.72	93.70	93.35	89.45	57.81
PM24h	45.57	42.45	97.63	93.46	93.15	89.45	57.96

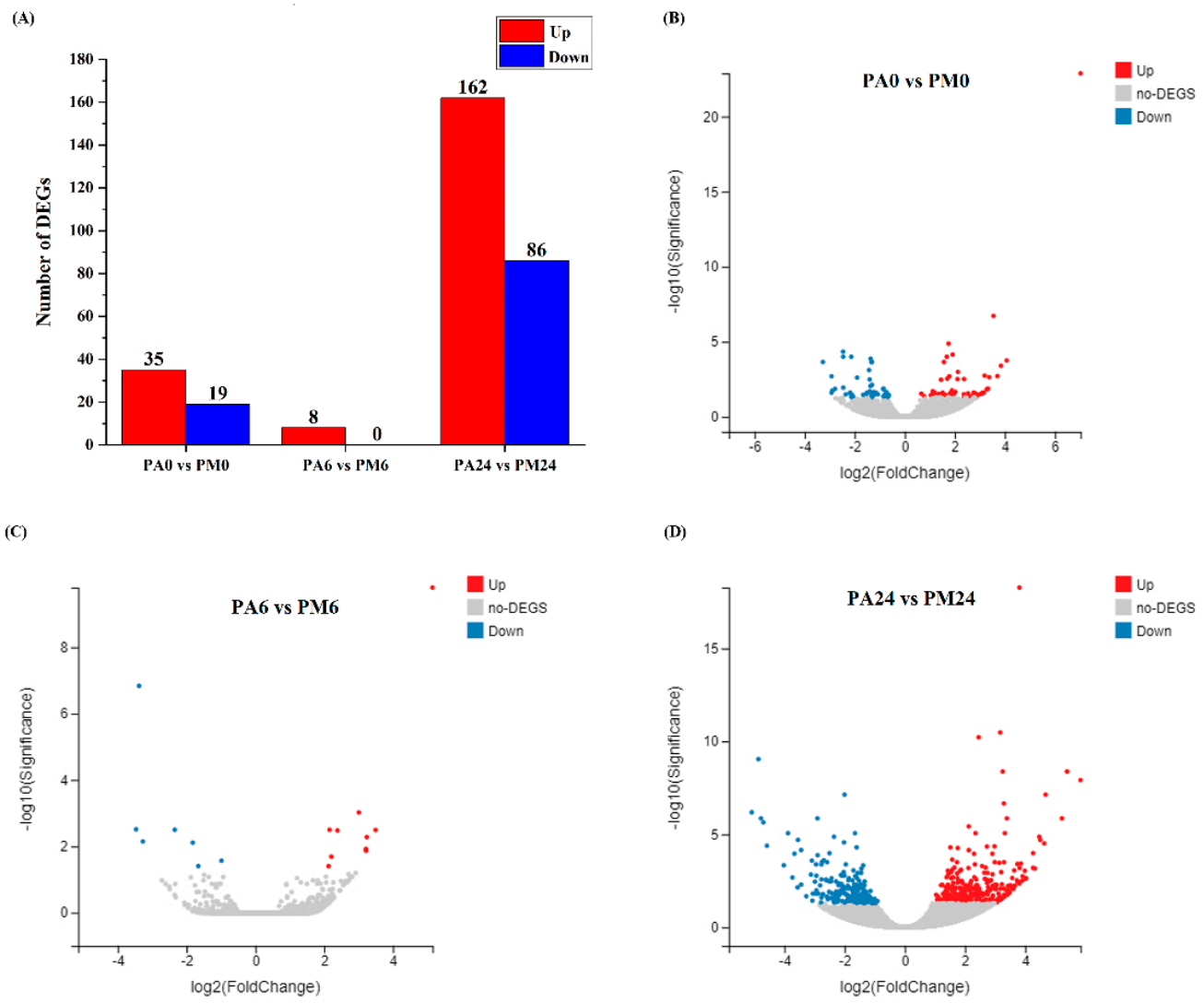

Figure 2. An overview of differentially expressed genes (DEGs). (**A**) Up- and downregulation of differentially expressed genes (**B**–**D**). Volcano plot showing the differentially expressed genes. The x-axis represents the log2(fold change) of the values, and the y-axis represents the significance value after –log10(significance). Red shows upregulated DEGs, blue shows downregulated DEGs, while grey represents no DEGs.

3.4. GO and KEGG Enrichment Analysis

The GO analysis was used to classify biological functions into three sub-classes, i.e., molecular function, biological process, and cellular components. The comparison of Bungo/PM0 vs. Bungo/PA0, Bungo/PM6 vs. Bungo/PA6, and Bungo/PM24 vs. Bungo/PA24 represents 84, 14, and 361 genes, respectively. In terms of biological processes, GO terms enriched with DEGs included metabolic and cellular processes. The most enriched terms for the cellular component were cell, membrane, and membrane parts. While in the case of molecular functions, GO terms enriched with DEGs included catalytic activity and binding activity (Figure 3). Furthermore, KEGG pathway enrichment analysis showed a total of 127 significantly enriched pathways (File S3). The plant hormone signal transduction (127 DEGs), ribosome (132 DEGs), circadian rhythm–plant (58 DEGs), and MAPK signaling pathway–plant (114 DEGs) were discovered to be significantly enriched among comparison groups (File S4).

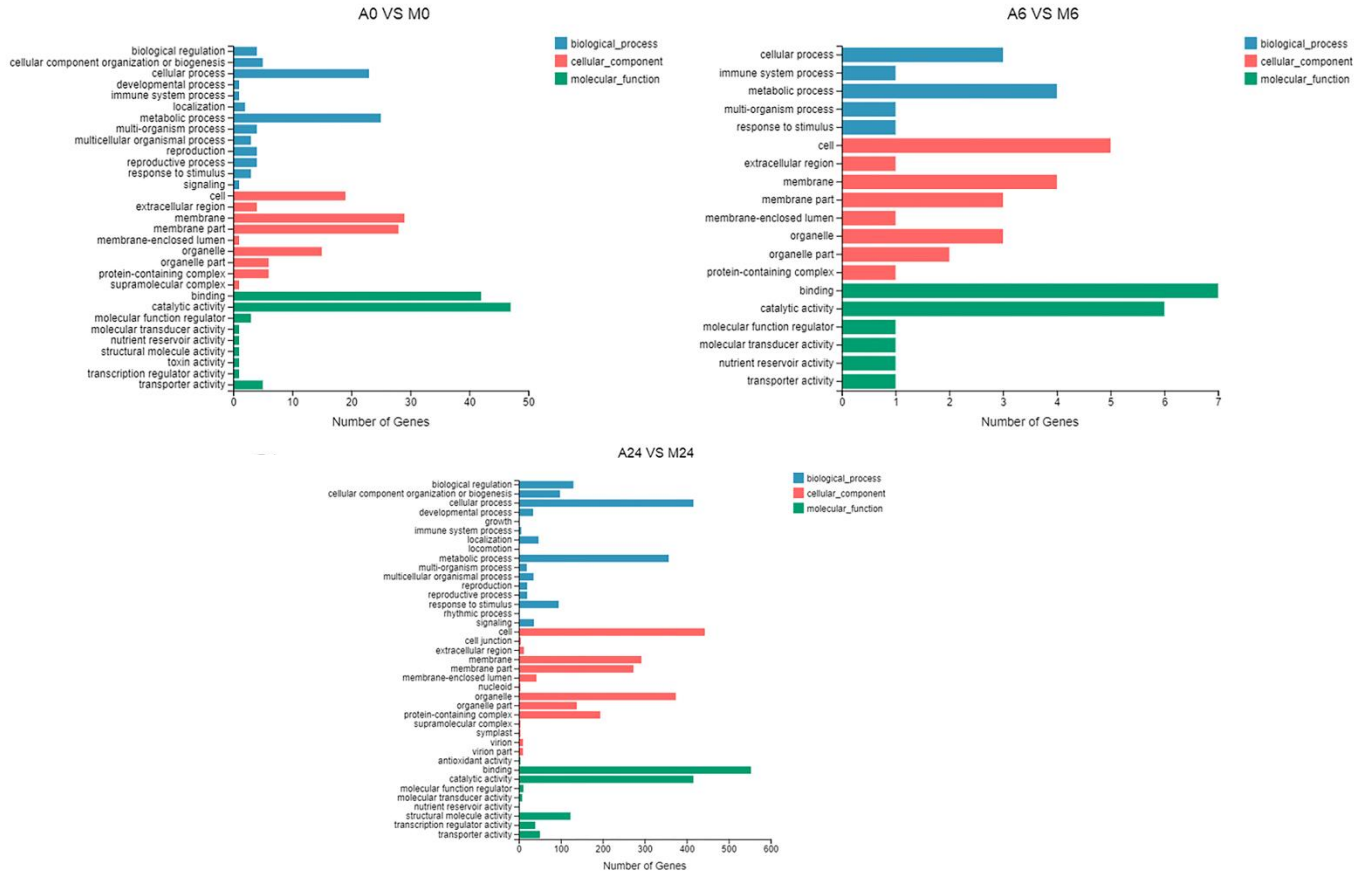

Figure 3. Gene Ontology (GO) analysis between cold-sensitive (Bungo/PM) and cold-tolerant (Bungo/PA) grafted plants under cold stress at 0, 6, and 24 h, showing the abundance of differentially expressed enriched GO terms. The most enriched terms of 'biological process, cellular component, and molecular function' are shown.

3.5. DEGs Related to Plant Hormone Signaling Transduction Pathway

Earlier research has demonstrated that plant hormones such as auxin (IAA), cytokinin (CK), gibberellin (GA), and abscisic acid (ABA) and others play a vital role in modulating plant responses to abiotic stresses such as low-temperature stress. Thus, we studied the regulation and expression patterns of the DEGs related to plant hormones. According to the KEGG analysis, the most enriched pathway is plant hormone signal transduction (Figure 4). A total of 126 DEGs were identified to be involved in auxin (IAA), abscisic acid (ABA), gibberellin (GA), cytokinin (CK), jasmonic acid (JA), brassinosteroid (BR), and ethylene (ET) mediated pathways. Figure 4B depicts gene expression profiles for the

various plant hormones involved in this study. These DEGs display distinct expression patterns throughout time, revealing a complex mechanism involving phytohormones in cold tolerance. Auxins have been conferred recently regarding their possible functions in cold stress tolerance. A total of 29 auxin-related genes were found to be differentially expressed, including 10 auxin response factors (*ARF*), 8 AUX/indole-3-acetic acids (*Aux/IAA*), 1 Gretchen Hagen 3s (*GH3*s), 8 small Auxin-Up RNAs (*SAUR*s), and 2 were involved in transport inhibitor response 1 (*TIR1*). In the present study, five *SAUR* genes were differentially expressed. *SAUR32* (LOC103322112), probable complex I intermediate-associated protein 30 (LOC103327582), *auxin-induced protein 6B* (LOC103321273), and *auxin-induced protein 15A-like* (LOC103335842) were upregulated, while another auxin response factor 17 (LOC103332948) and auxin response factor 18-like (LOC103342137) were downregulated. Seventeen genes involved in ABA signaling were found to be differentially expressed. One *SnRK2* (LOC103342612), *PYL4* (LOC103341293), *PP2C24* (LOC103321870), *PP2C2 56-like* (LOC103340823) were upregulated in both cold-sensitive and cold-tolerant graft combinations denoting that it could play a crucial role under cold stress. While, ABSCISIC ACID-INSENSITIVE-like protein *ABF5* (LOC103320341) and *ABF7* LOC103335361) were upregulated in the leaves of both cultivars after cold stress. Regarding brassinosteroid, we found that only squamosa promoter-binding-like protein 3 (LOC103326054), probable serine/threonine-protein kinase At4g35230 (LOC103331397), and a cyclin-D3-1 protein *CYCD3* (LOC103335336) were identified to be downregulated. Both rootstocks shared 7 of the 10 DEGs involved with the JA signaling pathway, and their expression patterns were comparable as well. However, we found that *TIFY 6B-like protein* (LOC103321427) was shown to be specifically increased at 24 h treatments. These alterations in JA signaling pathway imply that ZIM motif family proteins may express particularly to late (24 h) cold stress. Eight of the 14 DEGs were highly enriched in the pathway mediated by cytokinin. We noticed upregulation of histidine kinase 3 (LOC103322084) specifically in cold-sensitive; when cold stress was extended, the expression increased. Moreover, we found that two-component response regulator-like *APRR2* (LOC103328914) and protein PHR1-LIKE 1 (LOC103318708) were upregulated. Two histidine-containing phosphotransfer proteins *AHP* (LOC103333829, LOC103344697) were downregulated after 24 h of cold stress in both rootstocks.

3.6. DEGs in Response to Circadian Rhythm–Plants Pathway

To identify the molecular mechanisms involved in cold tolerance in Japanese apricot, the DEGs in the circadian rhythm pathway was mapped, and their expression patterns in various compartments were retrieved (Figure 5). Two zinc finger proteins (LOC103323323, LOC103324540), one transcription factor *TCP11* (LOC103342154) were found to be upregulated. The expression of an uncharacterized protein (LOC103330638) was specific to 6 h. In both rootstocks, we found that *TC-bHLH74* (LOC103320500), *TC-TCP9-like* (LOC103337396) were also upregulated in 6 h and either downregulated or did not differentially express at 24 h of cold stress. The early expression of TC genes in the Japanese apricot might be regarded as a cold response.

3.7. DEGs in Response to MAPK Signaling Pathway

In the current study, MAPK signaling pathway, ABA, JA, and ethylene routes were enriched in differentially expressed genes that displayed significantly different gene expression levels associated with abiotic stress defense (Figure 6). Moreover, a total of 32 DEGs were found to be enriched in pathogen infection signaling. Of these, four *WKRYs* (*WRKY57*, *WRKY40*, *WRKY50*, *WRKY7*) were upregulated in both cultivars, except *WRKY23* and *WRKY65,* which was specific to 6 h. Additionally, abscisic acid receptor *PYL2* (LOC103320543), receptor-like protein 12 (LOC103319054), and *PP2C 24* (LOC103321870) were also upregulated. Additionally, two MAPK-like genes, i.e., mitogen-activated protein kinase *NPK1* (LOC103321218) and mitogen-activated protein kinase homolog *NTF3* (LOC103320886), were downregulated at 24 h and upregulated in 6 h.

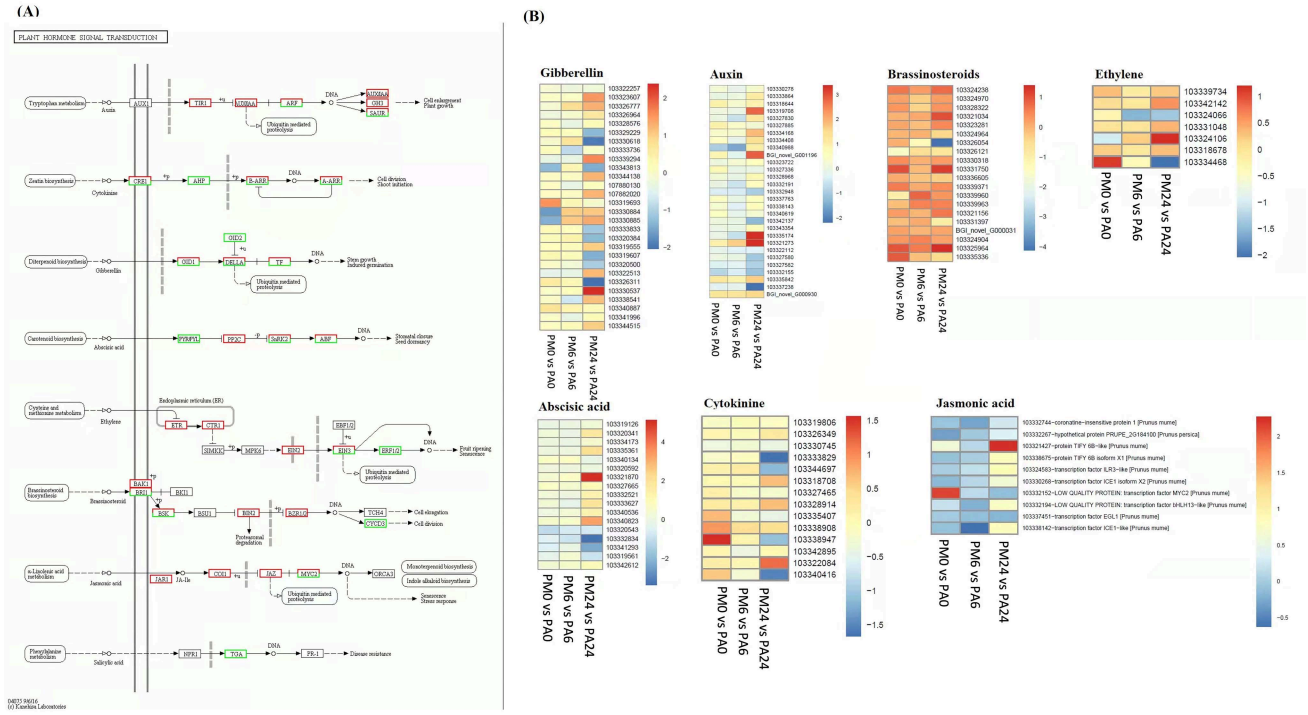

Figure 4. KEGG analysis exhibiting the enrichment of DEGs in the plant hormone signal transduction pathway. (**A**) Heatmap shows the expression of DEGs in cold-sensitive (Bungo/PM) and cold-tolerant (Bungo/PA) grafted combinations associated with different hormones like auxin, cytokinin gibberellin, abscisic acid, jasmonic acid, brassinosteroid, and ethylene. The bar represents the expression level of each gene as indicated by blue (lower expression) and dark yellow (higher expression) (**B**).

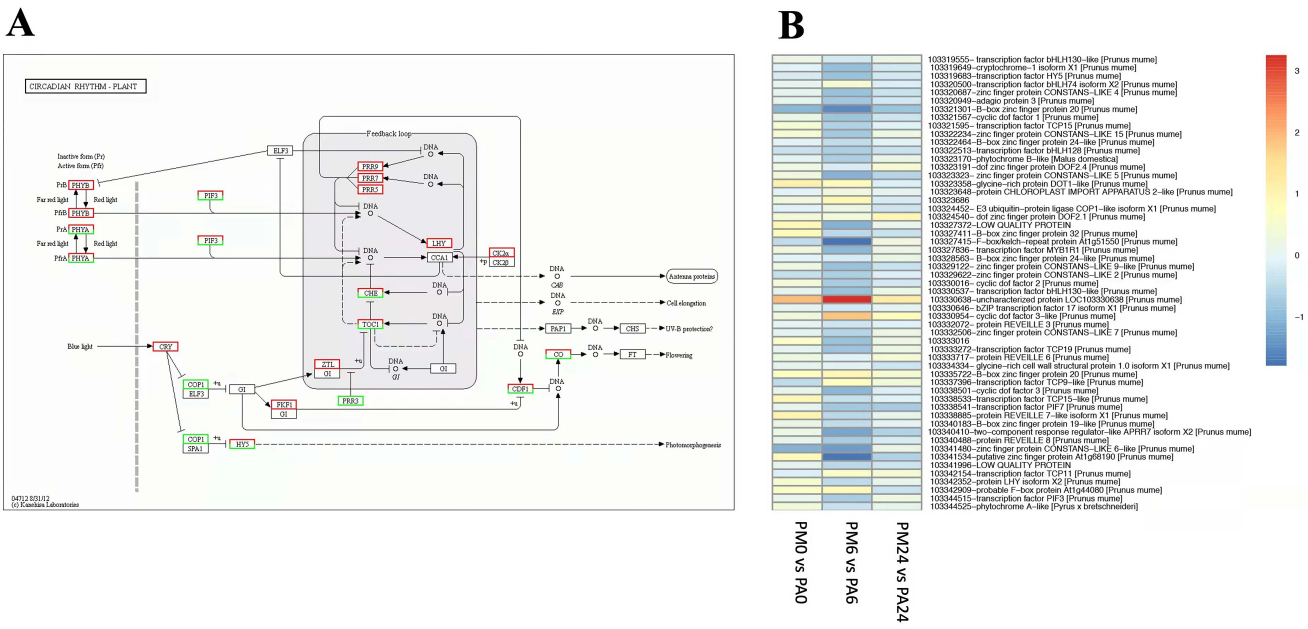

Figure 5. KEGG analysis exhibiting the enrichment of DEGs in "circadian rhythms" (**A**). Heatmap shows the expression of DEGs in cold-sensitive (Bungo/PM) and cold-tolerant (Bungo/PA) grafted combinations. The bar represents the expression level of each gene as indicated by blue (lower expression) and dark yellow (higher expression) (**B**).

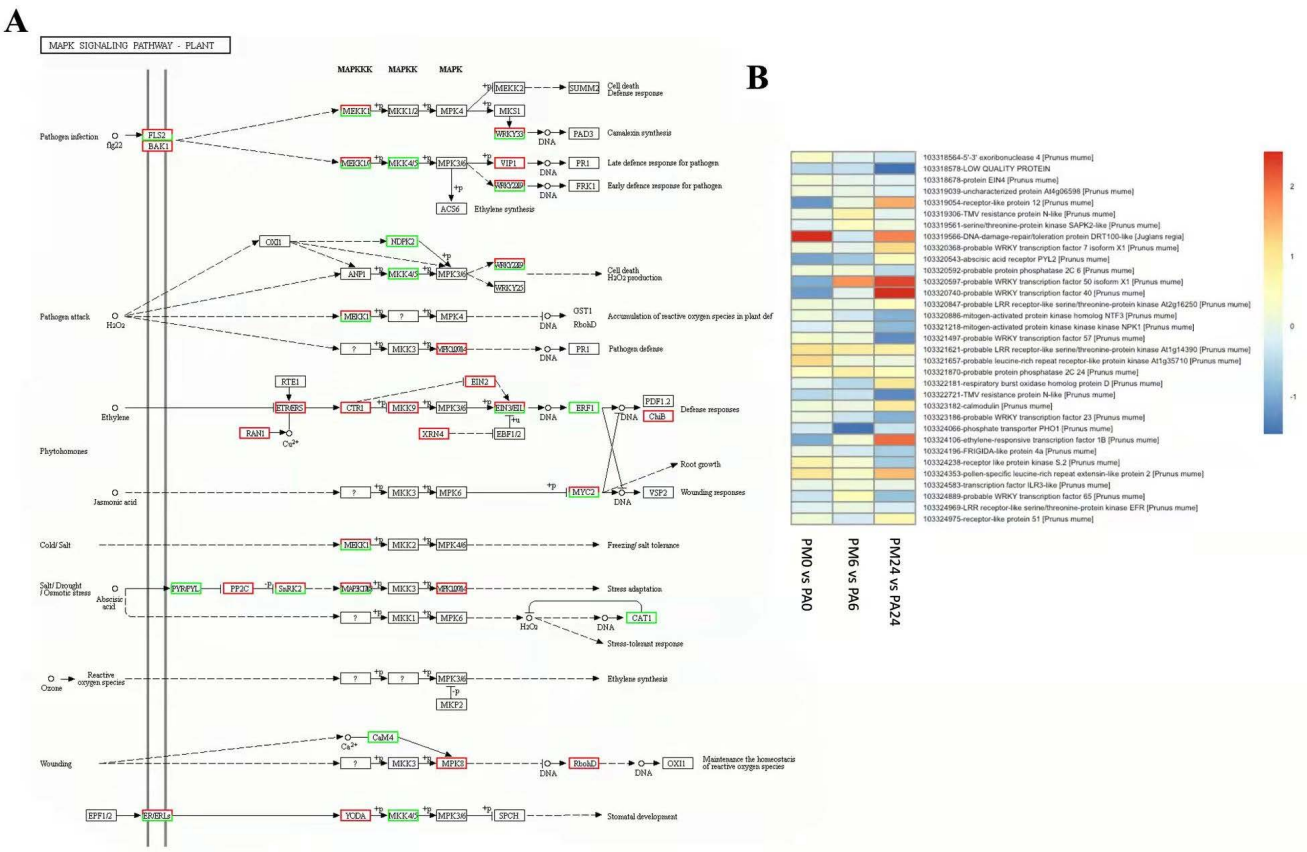

Figure 6. KEGG analysis exhibiting the enrichment of DEGs in the "MAPK signaling pathway" (**A**). Heatmap shows the expression of DEGs in cold-sensitive (Bungo/PM) and cold-tolerant (Bungo/PA) grafted combinations. The bar represents the expression level of each gene as indicated by blue (lower expression) and dark yellow (higher expression) (**B**).

3.8. DEGs in Response to Ribosome Pathways

Ribosomes are required for all living creatures because they serve as the location for protein translation. Ribosome biogenesis is a highly controlled and important biological activity. In the current investigation, the ribosome pathway was shown to be enriched in differentially expressed genes under cold stress (Figure 7). After 6 h of cold stress, the ribosome pathway was substantially more enriched in cold-sensitive rootstock than in cold-tolerant rootstock. In total, 132 DEGs were expressed between cold-sensitive and cold-tolerant rootstock at 6 and 24 h. Especially at the 24 h time point, most of the genes were downregulated in cold-tolerant rootstock relative to cold-sensitive rootstock.

3.9. qRT-PCR Validation of Gene Expressions

The expression patterns of 12 candidate annotated genes were chosen to verify RNA-Seq data using qRT-PCR (Figure 8). The primers were designed using Primer 5 software and are listed in File S5. This result demonstrates that the expression levels of selected genes were consistent with the RNA-seq data.

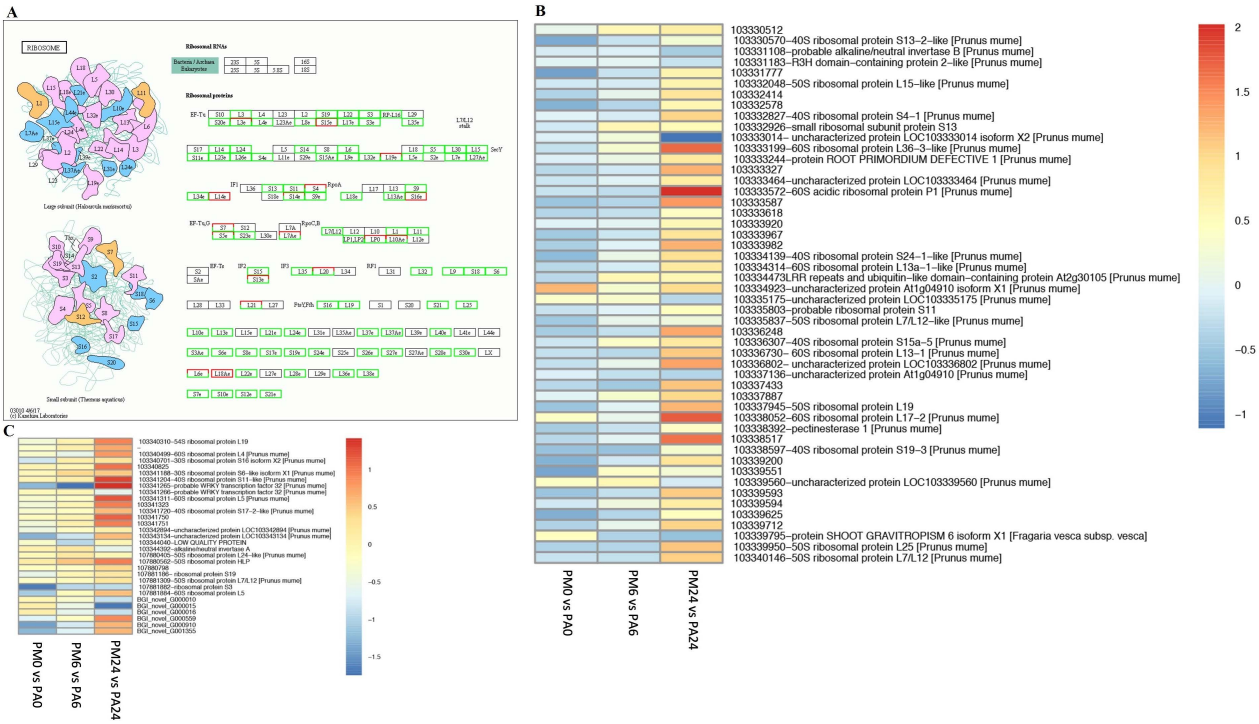

Figure 7. KEGG analysis exhibiting the enrichment of DEGs in the "ribosome pathway" (**A**). Heatmap shows the expression of DEGs in cold-sensitive (Bungo/PM) and cold-tolerant (Bungo/PA) grafted combinations. The bar represents the expression level of each gene as indicated by blue (lower expression) and dark yellow (higher expression) (**B**,**C**).

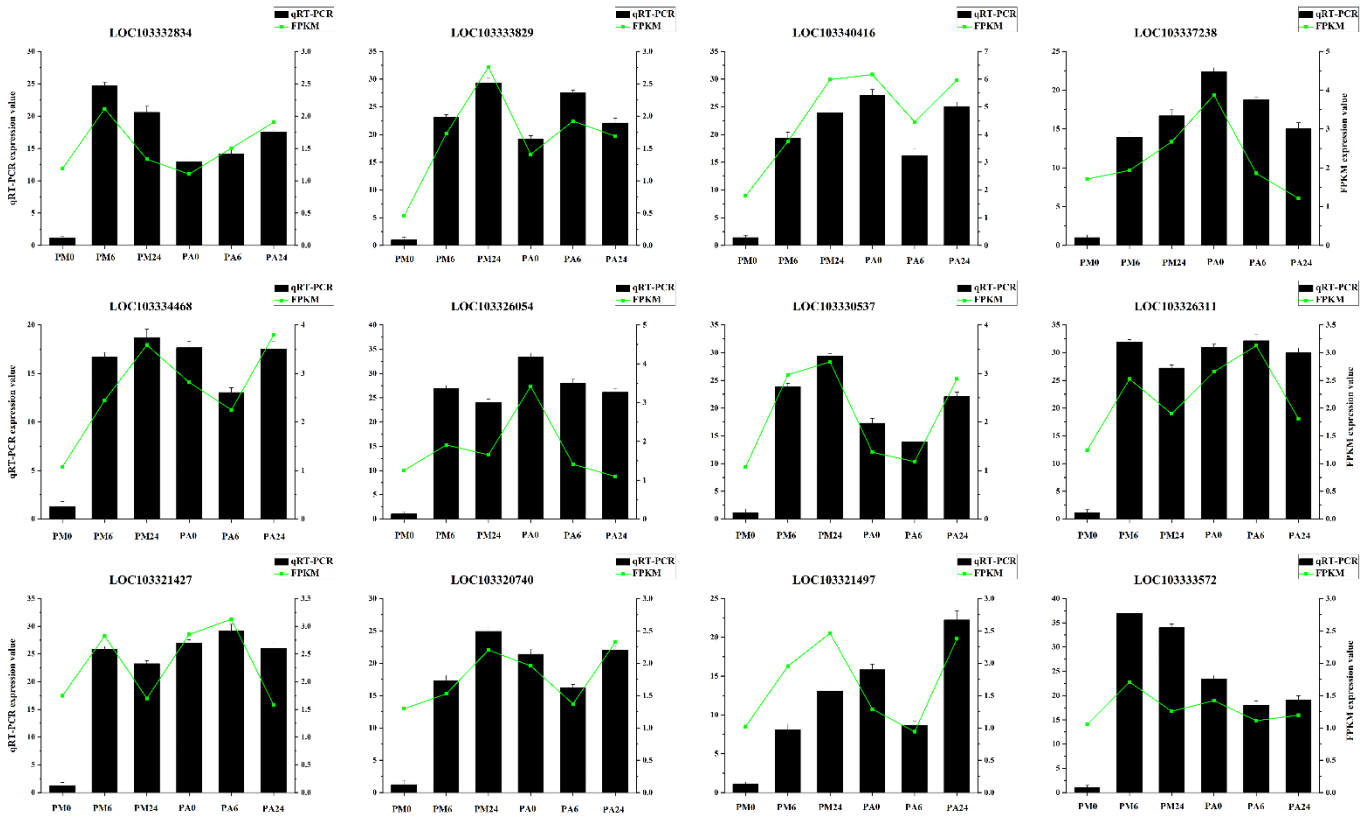

Figure 8. Validation of RNA-seq data by real-time quantitative RT-PCR. Column chart represents gene expression levels of qRT-PCR at 0, 6, and 24 h under cold stress. Error bars indicate the standard error as mean + SD.

4. Discussion

Grafting is a centuries-old horticultural practice that improves biotic and abiotic stress resistance [27]. Rootstocks can enhance stress resistance and increase yield potential; however, the physiological and molecular mechanisms underlying rootstock-induced phenotypic changes remain largely unknown [55,56]. Grafting of potential scion cultivars onto tolerant rootstocks is an advanced strategy to enhance the abiotic stress resistance of fruit plants. Two months after grafting, significant differences were found in the growth parameters of the 'Bungo' Japanese apricot grafted onto the different rootstocks. We further noticed that the morphological traits, including primary shoot length, leaf area, and overall biomass of 'Bungo' scion grafted on *P. armeniaca* (Bungo/PA) was noticeably reduced compared to that of *P. mume* (Bungo/PM) rootstock. Moreover, the scion morphology alterations were due to differences in rootstock characteristics. According to the best of our knowledge, the ability of rootstocks to improve the cold tolerance of Japanese apricot is not understood.

Cold stress negatively affects plant growth, development, and geographical distribution [57,58]. Cold tolerance is mediated by multiple gene expressions and various biochemical pathways. A deep investigation of molecular mechanisms such as hormonal regulation, MAPK signaling, ribosome and circadian rhythm pathways under low-temperature stress provides an opportunity to explore cold tolerance and investigate the regulation of the genes involved in this pathway. In this study, two graft combinations, Bungo on cold-sensitive (*P. mume*) and Bungo on cold-tolerant (*P. armeniaca*) rootstocks, were used in transcriptome analysis to explore the biological mechanisms responsible for cold tolerance in Japanese apricot. Several studies have reported genome-wide transcriptional processes during plants exposed to cold stress in various crops, including *Fragaria*×*ananassa*, *Canarium album, Saussurea involucrate,* and hulless barley [59–62]. In the current study, RNA-Seq was employed to perform Illumina sequencing, and a total of 310 genes were retrieved after a cutoff value $p < 0.01$. A cluster analysis of the selected genes was performed to examine the expression pattern and changes under cold stress.

Similarly, proline and soluble carbohydrates have been shown to protect plants against cold-induced oxidative damage. Both plants of grafted combinations under cold stress showed an increase in proline contents; however, the cold-tolerant genotype "apricot" induced a higher synthesis in control and stress conditions, with results in line with those previously found in rice [63]. Proline removes excess H^+, regulates oxidative respiration, accumulates carbon and nitrogen, and improves protein water-binding capacity via hydrophobic interactions with surface residues [64]. As previously described, soluble sugars may accumulate in stressed plants and serve as osmoprotectants against freezing/dehydration damage [65]. The cold-tolerant rootstock 'apricot' induced an increment substantially in soluble sugars, while the sensitive rootstock 'mume' induced a decrease. Morsy [66] showed that rising soluble sugars under low temperatures might be a helpful marker for cold tolerance in rice. The higher antioxidant enzyme activities such as SOD, POD, and CAT was found in the leaves of Bungo grafted on tolerant genotype that might participate against ROS generation [67], protecting from oxidative damage through antioxidant activity [68]. The biochemical examination of the Bungo/PM cold-sensitive plant combination showed that the lower activity of CAT, POD, and SOD might limit the efficiency of the plant cells to scavenge harmful free radicals. These results suggested that increased antioxidant activity seems to be essential for cold stress tolerance. The higher rate of CAT, SOD, and POD activity enhances cold-induced oxidative stress tolerance [69,70].

Plant hormones serve as signaling molecules involved in complicated physiological activities in plants, including growth, development, and morphogenesis [71]. Previous studies have shown that plant hormones function as key regulators of cold responses [28,29]. In this regard, DEGs were shown to be enriched in these signaling related pathways. A total of 125 DEGs associated with plant hormones were identified, and their expression patterns were analyzed in this study. IAA is a plant hormone that significantly impacts

growth and stress response in association with other hormonal pathways [72–74]. Recently, auxins have been addressed concerning their putative role in cold stress tolerance.

Several gene families, including the Auxin response factor (*ARF*), Gretchen Hagen 3 (*GH3*), Auxin/indole-3-acetic acid (*Aux/IAA*), and small Auxin-Up RNA (*SAUR*), are essential components of the IAA signaling pathway. These genes stimulate plant growth and response to cold stress [75,76]. The upregulation of *GH3* in both cold-sensitive and cold-tolerant plant combinations in specifically 24 h is suggestive of such a role (Figure 4B). The *GH3* gene was recently investigated for its function and response to biotic and abiotic stresses in various plant species, including cold stress [20,77]. ABA induces multiple changes in molecular, physiological, and developmental progressions, resulting in plant adaptation to the stress environment [78]. It is classified as an intracellular messenger that may play a critical role in cold stress signaling [79,80]. Additionally, the increased expression of ABA specific genes, i.e., *SnRK2* (LOC103319561, LOC103342612), *PYRPYL* (LOC103341293), *PP2Cs* (LOC103321870, LOC103327665, LOC103340536), and *ABF* (LOC103340134) clearly demonstrates that both grafted plants detect cold stress and ABA signaling related genes are involved in cold stress. The increased levels of JA have been reported in response to cold exposure [81]. The *JAZ* proteins are the most critical repressors of JA responsive genes; the upregulation of the *JAZ* genes (LOC103321427, LOC 103338675) in cold-sensitive combinations (Bungo/PM) contributes to enhancing cold sensitivity. In the most recent study [20], the cold-sensitive maize inbred line (Q319) had lower tolerance due to upregulation of *JAZ* genes at the seedling stage, which is also one of the reasons for increasing cold sensitivity. These findings show that phytohormones including IAA, ABA, and JA have a role in cold tolerance in Japanese apricot.

The circadian clock triggered diurnal rhythmic gene expression to cope with environmental change fluctuations. Organisms have evolved adaptation mechanisms that are connected with environmental variations such as light/dark cycles and temperature fluctuations, exhibiting a significant change in physiology and metabolism in most organisms occurring between the day and night cycles [82,83]. In the current study, we also observed that the circadian rhythm–plants pathway was significantly enriched. In *Arabidopsis*, it has been shown to be influenced by cold stress and its function in cold acclimation [84–86]. Previous studies also reported that the *DOF* genes are involved in multiple physiological activities, such as plant growth and development, stress sensing and response, and circadian cycles [87–89]. In the current study, *cyclic DOF factor 3-like* (LOC103330954), *DOF zinc finger protein DOF2.1* (LOC103324540), and *DOF zinc finger protein DOF 2.4* (LOC103323191) genes were revealed to be upregulated at 6 h in Bungo/PM grafted combination suggesting that they might have a role in the early cold response. Moreover, the downregulation of *putative zinc finger protein At1g68190* (LOC103341534) and *B-box zinc finger protein 20* (LOC103321301) genes at 24 h in Bungo/PM is also one of the reasons for increased cold-sensitivity. In contrast, C2H2 zinc finger proteins enhance cold resistance by directly regulating downstream cold-related genes in plants [90].

Corrales [91] reported that overexpression of the *SlCDF1* and *SlCDF3* genes in Arabidopsis plants enhanced drought and salt tolerance compared to the wild type. The relatively higher upregulation of *PRR5*, *PRR7*, *PRR9* in both rootstocks in 6 and 24 h suggested that these genes may be necessary components of the circadian clock and alterations in their expression influence further the output pathways as well as the capability of plants to respond against abiotic stress [92]. The studies on *Solanum lycopersicum, Lolium perenne*, and *Elymus nutans* have revealed that cold stress may also affect circadian rhythm by controlling expression of abiotic stress-responsive genes, as well as biosynthesis and signaling downstream of stress response hormones [93–96].

In plants, *MAPKs* control gene expression by transducing second messengers and hormone signals to help adaptation under various stresses [97,98]. Likewise, the MAPK signaling pathway was significantly enriched in our study, indicating that this pathway is involved in cold stress responses. Calcium/calmodulin binds to *CRLK1* and stimulates it, after which *CRLK1* relates with *MEKK1*, resulting in *MAPK* activation under cold

tolerance [99]. In *A. thaliana*, some genes encoding *MPKs* (mitogen-activated protein kinases) were found to be increased in response to cold stress [100]. Moreover, we found a transcript *MPK8* (LOC103338711) that enhanced expression during cold stress. The phosphorylation levels of *MPK3*, *MPK4,* and *MPK6* were found to be substantially higher under low temperature [101]; furthermore, *MPK3* and *MPK6* might interact with *ICE1* to participate in low-temperature response [102]. A recent study [103] identified the *MAP3K* gene that played an essential role in cold stress signal transduction to improve FT in *Poncirus trifoliata*. In the current study, the upregulation of *WRKY7*, *WRKY40*, *WRKY50*, *WRKY57* indicates that they have a role in cold stress tolerance and their functions in alleviating oxidative stress [104]. In conclusion, the expression of genes associated with *MAPK* signaling pathway indicates that *MAPK* related genes may be playing a pivotal role in Japanese apricot cold stress responses.

5. Conclusions

In fruit crops, grafting of potential cultivars onto tolerant rootstock is an effective technique for improving tolerance to abiotic stresses, including cold stress. Low temperature severely affects the growth and development of *P. mume* and restricts its geographical distribution in the north of China. In the present study, an integrated transcriptome profile of two distinct scion/rootstock combinations (Bungo/*P. mume* and Bungo/*P. armeniaca*) was studied using RNA-seq to determine the gene expression levels and molecular mechanism of cold stress. Several differentially expressed genes (DEGs) were related to plant hormone signaling transduction, MAPK signaling, circadian rhythm–plants and ribosome pathways were retrieved, underlying the crosstalk of the cold response mechanism of Japanese apricot. Furthermore, lower levels of MDA and higher levels of osmo-protectants and antioxidant activities of SOD, CAT, and POD were observed in plants grafted onto *P. armeniaca* rootstock compared with *P. mume* rootstock, which helps plants to counter cold-induced oxidative damage. Overall, this study provides deepened information for future research on cold response mechanisms and is used for genetic improvement of cold tolerance in Japanese apricot.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7120572/s1, File S1. Detail of all biological repeats of transcriptome assembly; File S2. Expression pattern of the genes involved in different comparison; File S3. Total number of enriched pathways and their candidate genes identified in this study; File S4. Expression pattern values of the candidate genes of the significant pathways discussed in this study; File S5. Primer used in this study.

Author Contributions: Conceptualization, Z.G.; formal analysis, F.H., C.M., S.I., X.H., O.K.O. and Z.N.; methodology, F.H., S.I. and R.T.; resources, Z.N.; software, S.I., T.S. and R.T.; supervision, Z.G.; writing—original draft, F.H., C.M. and S.I.; writing—review and editing, S.I., T.S., U.K. and Z.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Key Research and development Program of China (2020YFD1000203), Jiangsu Belt and Road innovation cooperation project: Cooperative development of overseas application of molecular breeding technology in horticultural crops (BZ2019012), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) for funding this research, materials collection.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is submitted to NCBI-SRA under the accession number SRR171550655-72.

Acknowledgments: Thanks to all researchers for their contribution in this work.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

MDA	malondialdehyde
SOD	superoxide dismutase
POD	peroxidase
CAT	catalase

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