



Article

Transcriptomics Profiling of *Acer pseudosieboldianum* Molecular Mechanism against Freezing Stress

Zhiming Han ^{1,2,†}, Xiangzhu Xu ^{3,†}, Shikai Zhang ¹, Qiushuang Zhao ¹, Hanxi Li ¹, Ying Cui ², Xiao Li ², Liran Wang ², Su Chen ¹  and Xiyang Zhao ^{2,*}

¹ State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, Harbin 150040, China

² Jilin Provincial Key Laboratory of Tree and Grass Genetics and Breeding, College of Forestry and Grassland Science, Jilin Agricultural University, Changchun 130118, China

³ College of Veterinary Medicine, Jilin University, Changchun 130062, China

* Correspondence: xiyangz@jalu.edu.cn

† These authors contributed equally to this work.

Abstract: Low temperature is an important environmental factor that affects the growth and development of trees and leads to the introduction of failure in the genetic improvement of trees. *Acer pseudosieboldianum* is a tree species that is well-known for its bright red autumn leaf color. These trees are widely used in landscaping in northeast China. However, due to their poor cold resistance, introduced *A. pseudosieboldianum* trees suffer severe freezing injury in many introduced environments. To elucidate the physiological indicators and molecular mechanisms associated with freezing damage, we analyzed the physiological indicators and transcriptome of *A. pseudosieboldianum*, using kits and RNA-Seq technology. The mechanism of *A. pseudosieboldianum* in response to freezing stress is an important scientific question. In this study, we used the shoots of four-year-old *A. pseudosieboldianum* twig seedlings, and the physiological index and the transcriptome of *A. pseudosieboldianum* under low temperature stress were investigated. The results showed that more than 20,000 genes were detected in *A. pseudosieboldianum* under low temperature (4 °C) and freezing temperatures (−10 °C, −20 °C, −30 °C, and −40 °C). There were 2505, 6021, 5125, and 3191 differential genes (DEGs) between −10 °C, −20 °C, −30 °C, −40 °C, and CK (4 °C), respectively. Among these differential genes, 48 genes are involved in the MAPK pathway and 533 genes are involved in the glucose metabolism pathway. In addition, the important transcription factors (MYB, AP2/ERF, and WRKY) involved in freezing stress were activated under different degrees of freezing stress. A total of 10 sets of physiological indicators of *A. pseudosieboldianum* were examined, including the activities of five enzymes and the accumulation of five hormones. All of the physiological indicators except SOD and GSH-Px reached their maximum values at −30 °C. The enzyme activity of SOD was highest at −10 °C, and that of GSH-Px was highest at −20 °C. Our study is the first to provide a more comprehensive understanding of the differential genes (DEGs) involved in *A. pseudosieboldianum* under freezing stress at different temperatures at the transcriptome level. These results may help to clarify the molecular mechanism of cold tolerance of *A. pseudosieboldianum* and provide new insights and candidate genes for the genetic improvement of the freezing tolerance of *A. pseudosieboldianum*.

Keywords: *Acer pseudosieboldianum*; freezing stress; RNA-seq; DEGs



Citation: Han, Z.; Xu, X.; Zhang, S.; Zhao, Q.; Li, H.; Cui, Y.; Li, X.; Wang, L.; Chen, S.; Zhao, X. Transcriptomics Profiling of *Acer pseudosieboldianum* Molecular Mechanism against Freezing Stress. *Int. J. Mol. Sci.* **2022**, *23*, 14676. <https://doi.org/10.3390/ijms232314676>

Academic Editor: Bartolome Sabater

Received: 8 October 2022

Accepted: 17 November 2022

Published: 24 November 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Maple is a perennial deciduous tree or shrub of the genus maple in the family *Aceraceae*, with a natural distribution in Asia, Europe, North America, and the northern edge of Africa [1–3]. China is the main distribution area of maple species with rich genetic material resources, with more than 150 species of the genus maple, accounting for more than half of the world's maple resources. This is significant for research into the evolutionary history of maple species [4,5]. In past years, these species of the genus maple have been widely

introduced, domesticated, and utilized, due to their adaptability, resistance, ornamental value, and economic value [6–8]. Maple is widely used in the timber and pharmaceutical industries. It is a well-known modern landscape species, with large trees, beautiful foliage, colorful leaves, peculiar fruit shapes, and rich genetic variation, and it occupies an important position in urban ecosystems and landscape construction [9,10].

Through previous studies, extracts of *Acer* roots, leaves, bark, fruits, and seeds have been found to contain rich nutrients, such as amino acids, fatty acids, and mineral elements, as well as some key physiologically active substances, such as triterpenoids, chlorogenic acids, and neuronics [11]. In addition to their medicinal value, many *Acer* species have a rich variety of leaf color types during growth and development, such as from green to yellow (such as *A. catalpifolium* Rehde. and *A. truncatum* Bunge) and green to red (such as *A. mandshuricum* Maxim. and *A. pseudosieboldianum*). In addition, *Acer* species have a rich phenotypic variation in leaf shape, and three-, five-, and seven-leaved simple leaves can be found in *Acer* species, laying the foundation for further genetic improvement of *Acer* species [12–14].

However, most *Acer* trees are in danger of extinction due to frequent human activities and pests. Currently, most *Acer* tree species, such as *A. campestre* L., *A. yangbiense* Y.S.Chen & Q.E.Yang, and *A. miaotaiense* P.C. Tsoong, are listed as endangered by the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species, and these *Acer* species are also listed as national protected plants in China [15,16]. The valuable deciduous tree species *A. pseudosieboldianum* ($2n = 2x = 16$) is only fragmented in northeastern China, the Russian Far East, and the northern half of the Korean Peninsula [17,18]. It is regarded as one of the most promising native trees for landscaping in northeastern China, due to its use as an essential leaf plant, its gorgeous winged fruit, and its intensely crimson leaves in autumn.

Hypothermia, as an abiotic stress, not only affects the geographical distribution of many important crops, but also negatively affects crop yield and quality every year [19,20]. Hypothermia stress is classified as cold ($0\text{--}15\text{ }^{\circ}\text{C}$) and freezing ($<0\text{ }^{\circ}\text{C}$) and has a significant impact on plant survival and geographic distribution [21]. A large number of plant species grow in tropical and subtropical regions, and most of them are sensitive to cold. However, plants can increase their frost tolerance through cold acclimation [22,23].

Previous studies have shown that freezing stress can trigger the activation of defense systems in plants. The peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) protective enzyme activity is enhanced, reducing the accumulation of reactive oxygen species (ROS) and reducing the freezing stress damage to plants [24]. In plant endogenous hormones, abscisic acid (ABA) and proline act as important regulators of plant response to adversity stress, participating in this defense process. ABA is involved in plant growth and development regulation and responses to different environmental stresses, and freezing stress can promote the accumulation of ABA [25]. In terms of molecular regulation, the response of plants to freezing stress is mainly through the sensing and transduction of low temperature signals by cells to regulate gene expression, thereby regulating different metabolic pathways and signal transduction pathways, and responding at the transcriptional and translational levels [26].

There are thousands of genes that regulate these metabolites through upregulation and downregulation in freezing stress, and researchers have found that cold environments cause numerous alterations in the physiology and gene expression patterns that include carbohydrate metabolism, molecular chaperones, antifreeze proteins, signal transduction (receptor kinases, phosphatases, and Ca^{2+} binding proteins), and regulatory proteins [27–30]. The mechanisms of woody plant responses to freezing stress have drawn increased attention in recent years. By using transcriptomic and multi-omics analysis, it was possible to identify numerous cold-responsive genes that are involved in photosynthesis, calcium signaling, ABA homeostasis and transport, and antioxidant defense systems. For instance, the transcriptome alterations of *P. simonii* under cold stress were investigated. Microarray analysis identified many cold response genes involved in photosynthesis, Ca^{2+} signaling,

ABA homeostasis and transport, and antioxidant defense systems [31]. The responses of *P. tremula* L. and *P. tremuloides* Michxto cold stress have also been studied. Four different soluble carbohydrates (glucose, fructose, sucrose, and alginate) were found to accumulate rapidly under cold treatment, and proteomic analysis detected many important proteins in this process, such as chaperone proteins, dehydrins, and late-embryonic-development-enriched proteins [32,33].

In northeastern China, *A. pseudosieboldianum* is mainly found in the cold-temperate zone and can withstand temperatures below 30 °C in winter. It has unique adaptability and cold resistance compared with related *Acer* species [34,35]. *A. pseudosieboldianum* may be a potential resource of red-leaved maple trees for landscaping and future hybridization studies of the genus *Macrocarpa*. Currently, the species is listed as endangered by the Chinese government and is protected *ex situ*, making *A. pseudosieboldianum* extremely valuable for research aimed at development and utilization.

Low temperature is an important environmental factor that affects the growth and development of trees and leads to the introduction of failure in the improvement of tree genetics. To elucidate the physiological indicators and molecular mechanisms associated with freezing damage, we analyzed the physiological indicators and transcriptome of *A. pseudosieboldianum*, using kits and RNA-Seq technology. In this experiment, four-year-old *A. pseudosieboldianum* twigs were used as samples, and the genes of *A. pseudosieboldianum* under freezing stress were studied through transcriptome.

2. Results

2.1. Measurement and Analysis of *A. pseudosieboldianum* Physiological Indicators

The production of reactive oxygen species is unavoidable in the normal life activities of plants, while reactive oxygen species scavenging systems exist in plants to maintain normal physiological activities. When plants are under adverse conditions, such as low temperature or drought, the balance between the intracellular production and the scavenging of reactive oxygen species is disrupted, and the increase of reactive oxygen species leads to cell injury.

In this study, we measured the activities of SOD, POD, CAT, APX, and GSH-Px and measured ABA, MDA, H₂O₂, proline, and soluble sugar by the enzymatic activities of five enzymes and the degree of accumulation of five hormones and metabolites to observe how various physiological indicators accumulated under freezing stress at different temperatures (Figure 1). As shown in Figure 1, the activities or concentrations of the hormones reached their highest values under freezing stress at −30 °C, except for SOD and GSH-Px; SOD reached its highest activity under freezing stress at −10 °C, and GSH-Px reached its highest activity under freezing stress at −20 °C.

2.2. Illumina RNA-Seq and Align Analysis

The transcriptome sequencing analysis of 15 samples was completed and a total of 135.57 Gb clean data were obtained. The percentage of clean data of each sample reached 7 Gb, Q30 bases, and the GC content exceeded 92%. The GC content was more than 44%, and the genomic spectrum rate was more than 73%, reaching 87.19%, and 91.24%, respectively (Tables 1 and S1). The average unique mapped reads reached 84.57%, which is enough for subsequent bioinformatics analysis [27,32].

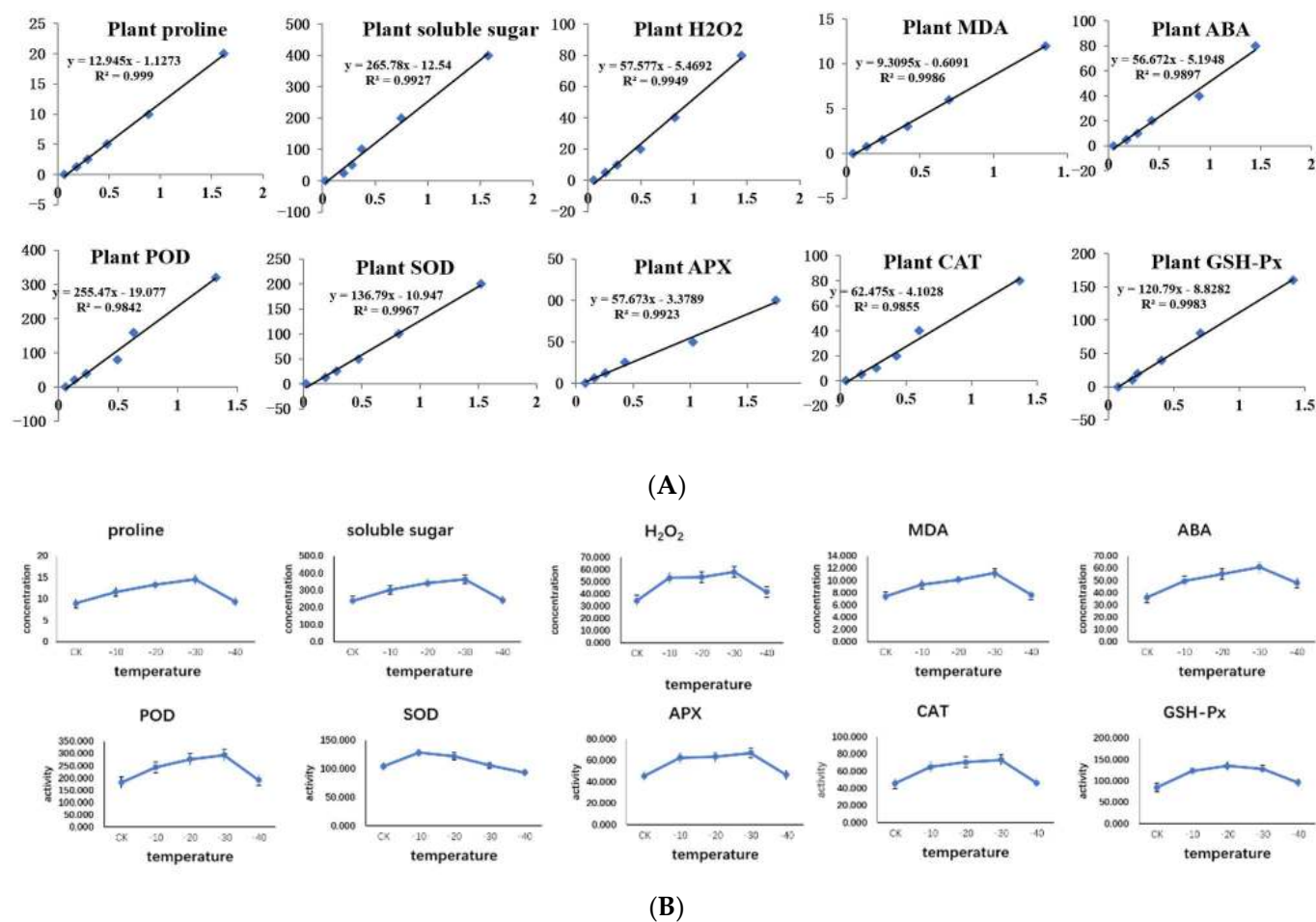


Figure 1. The expression of physiological indicators of *A. pseudosieboldianum* under freezing stress at different temperatures. (A) is the curve analysis of different physiological indicators to determine the accuracy of the test data; (B) is the activity and content expression of physiological indicators under freezing stress at different temperatures. These include the enzymatic activities of SOD, POD, CAT, APX and GSH-Px and the accumulation of ABA, MDA, H₂O₂, proline, and soluble sugar under freezing stress at different temperatures.

Table 1. Summary of the RNA-seq data collected from *A. pseudosieboldianum*.

Sample	Raw Reads	Clean Reads	Clean Base (G)	Q20 (%)	Q30 (%)	GC Content (%)	Reads Mapped	Unique Mapped
APCK-1	67,112,578	62,694,296	9.4	98.05	94.31	44.34	54,661,894 (87.19%)	51,783,721 (82.60%)
APCK-2	72,192,138	67,231,322	10.08	98.11	94.53	44.42	58,666,971 (87.26%)	55,645,093 (82.77%)
APCK-3	77,334,844	72,727,740	10.91	98.18	94.66	44.52	63,647,230 (87.51%)	60,429,351 (83.09%)
APA-1	69,972,072	64,699,338	9.7	98.16	94.61	44.58	57,852,215 (89.42%)	54,562,832 (84.33%)
APA-2	68,091,786	64,238,838	9.64	98.13	94.52	44.61	57,515,124 (89.53%)	54,348,737 (84.60%)
APA-3	66,558,226	62,252,698	9.34	98.19	94.72	44.65	55,850,429 (89.72%)	52,916,333 (85.00%)
APB-1	65,941,198	63,454,732	9.52	97.28	92.85	44.04	56,615,003 (89.22%)	53,874,843 (84.90%)
APB-2	52,211,874	50,641,818	7.60	97.27	92.80	43.83	45,193,337 (89.24%)	43,078,994 (85.07%)
APB-3	57,659,774	56,777,300	8.52	97.04	92.15	43.77	50,733,924 (89.36%)	48,444,786 (85.32%)
APC-1	61,411,604	54,530,820	8.18	98.21	94.78	44.48	49,423,885 (90.63%)	46,837,461 (85.89%)
APC-2	64,932,060	61,789,950	9.27	98.18	94.68	44.07	56,368,800 (91.23%)	53,583,580 (86.72%)
APC-3	67,066,254	63,155,790	9.47	98.15	94.59	44.15	57,620,465 (91.24%)	54,696,618 (86.61%)
APD-1	59,016,432	56,378,222	8.46	97.95	94.17	44.93	50,014,664 (88.71%)	47,167,630 (83.66%)
APD-2	49,445,182	46,892,166	7.03	98.21	94.77	44.87	41,665,473 (88.85%)	39,416,305 (84.06%)
APD-3	60,492,000	56,359,986	8.45	98.21	94.77	44.85	50,029,208 (88.77%)	47,315,447 (83.95%)

APCK was treated at 4 °C of *A. pseudosieboldianum*, APA at −10 °C, APB at −20 °C, APC at −30 °C, and APD at −40 °C. Reads mapped is the number of reads compared with the reference genome, and unique mapped is the only number of reads that is aligned with the reference genome.

2.3. Identification and Analysis of DEGs

In this experiment, DESeq2 was used to analyze the differentially expressed genes, and the total number of differentially expressed genes, the number of upregulated genes, and the number of downregulated genes in each group were counted (Table S2). By comparing the *A. pseudosieboldianum* of different periods, we studied the specific DEGs of freezing stress at different temperatures. At 4 °C, −10 °C, −20 °C, −30 °C, and −40 °C, and at 4 °C as the CK group, 2505 (996 upregulated genes and 1509 downregulated genes), 6021 (2587 upregulated genes and 3434 downregulated genes), 5125 (2095 upregulated genes and 3030 downregulated genes), and 3191 (1068 upregulated genes and 2123 downregulated genes) DEGs were identified, respectively, in comparison with the CK group (Figure 2C).

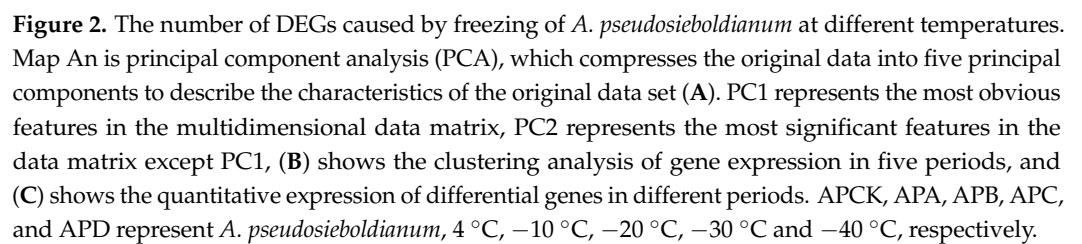
As shown in Figure 3, 870 common DEGs were detected under five different degrees of freezing stress, which may indicate that these DEGs were involved in the treatment of low temperatures at different low temperatures, and all participated in the same pathway in response to freezing stress. In addition, at −30 °C, the number of DEGs only (2002) was significantly higher than that of other groups, indicating that these genes may be related to the difference in cold tolerance of *A. pseudosieboldianum*.

In this study, the total RNA from the branch was used for qRT-PCR verification. As shown in Figure 3, the six DEGs showed similar expression patterns between qRT-PCR data and RNA-seq results, proving that RNA-seq data are highly reliable for further analysis.

2.4. Notes and Enrichment of *A. pseudosieboldianum* Transcriptome

To understand the function of DEGs found between the two cultivars, all DEGs and protein databases were searched, and then gene ontology (GO) analysis was carried out to evaluate the function of genes. Gene Ontology [36] (GO) is the international standard classification system of gene function. As a database established by the Gene Ontology Consortium, it aims to establish a language vocabulary standard that is suitable for various species, defines and describes the function of genes and proteins, and can be updated with the deepening of research. GO is divided into three parts: molecular function, biological process, and cellular component. In the GO terms of each treatment, the greater the proportion of DEGs with stronger freezing stress, the more changes that were made in the metabolic process (Figure 4). In the comparative analysis of the five groups, the DEGs of these GO terms were divided into “metabolic process”, “cell process”, and “response to stimulation”; in the cellular component, they were divided into “cell”, “cell part”, and “membrane”; and in molecular function, they were divided into “catalytic activity”, “binding”, and “transporter activity”.

In addition, to elucidate the metabolic pathways involved in freezing stress, 20 pathway items with the most significant enrichment were selected from the five freezing treatments of *A. pseudosieboldianum* to be displayed in the diagram (Figure 5). Among these top pathways, carbohydrate metabolism, galactose metabolism, fructose, and mannose metabolism are associated with many ascending/descending genes. In addition, in the comparison of the five groups of experimental samples, *A. pseudosieboldianum* was enriched in some pathways under all freezing stress, but there were some special pathways in each period, such as the synthesis and degradation of ketone bodies, which were significantly enriched only at −10 °C. The MAPK signaling pathway was significantly enriched under all freezing stress, ABC transport was enriched at −10 °C, −20 °C, and −40 °C, and Limonene and pinene degradation was enriched only at −40 °C. In short, many genes from different metabolic pathways are involved in the regulation of *A. pseudosieboldianum* freezing stress, and further study on the differential expression patterns of these pathways is of great significance in revealing the cold resistance of *A. pseudosieboldianum*.



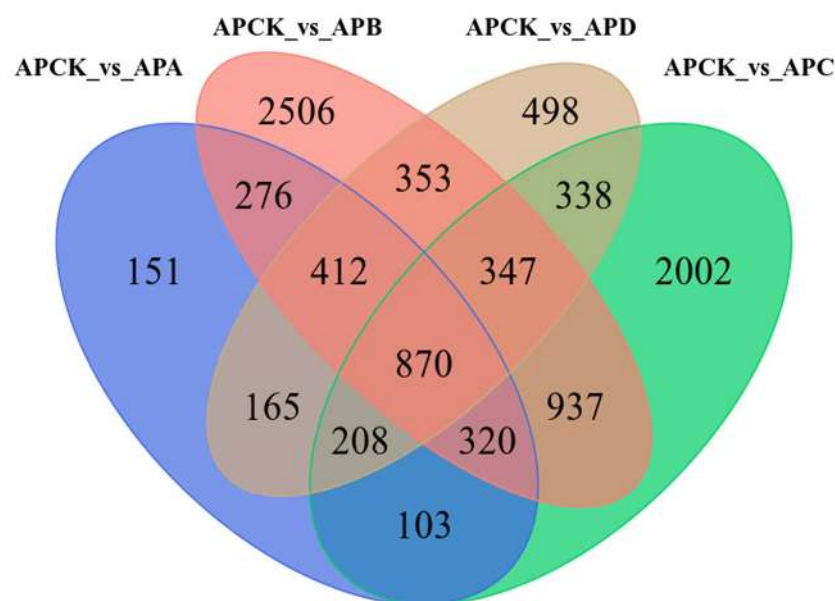


Figure 3. Wayne diagram of *A. pseudosieboldianum* and DEGs of CK (4 °C) under different temperature freezing stress. They provide a comparison between −10 °C treatment and 4 °C treatment, −20 °C treatment and 4 °C treatment, −30 °C treatment and 4 °C treatment, and −40 °C treatment and 4 °C treatment.

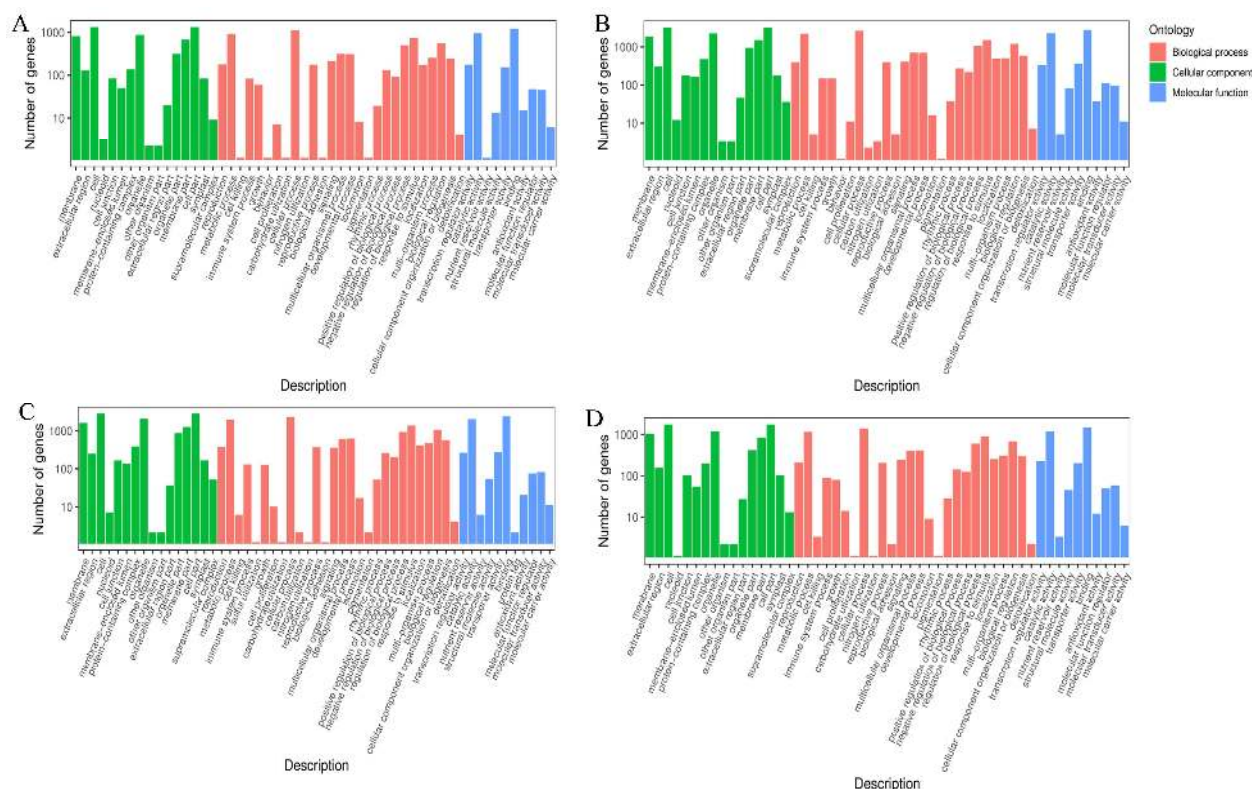


Figure 4. GO analysis of DEGs of *A. pseudosieboldianum* under freezing stress at different temperatures. The Y and X axes correspond to GO terminology and the number of DEGs. The (A) figure shows the comparison of −10 °C treatment and 4 °C treatment; the (B) diagram shows the comparison of −20 °C treatment and 4 °C treatment; the (C) diagram shows the comparison of −30 °C treatment and 4 °C treatment; and the (D) diagram shows the comparison of −40 °C treatment and 4 °C treatment.

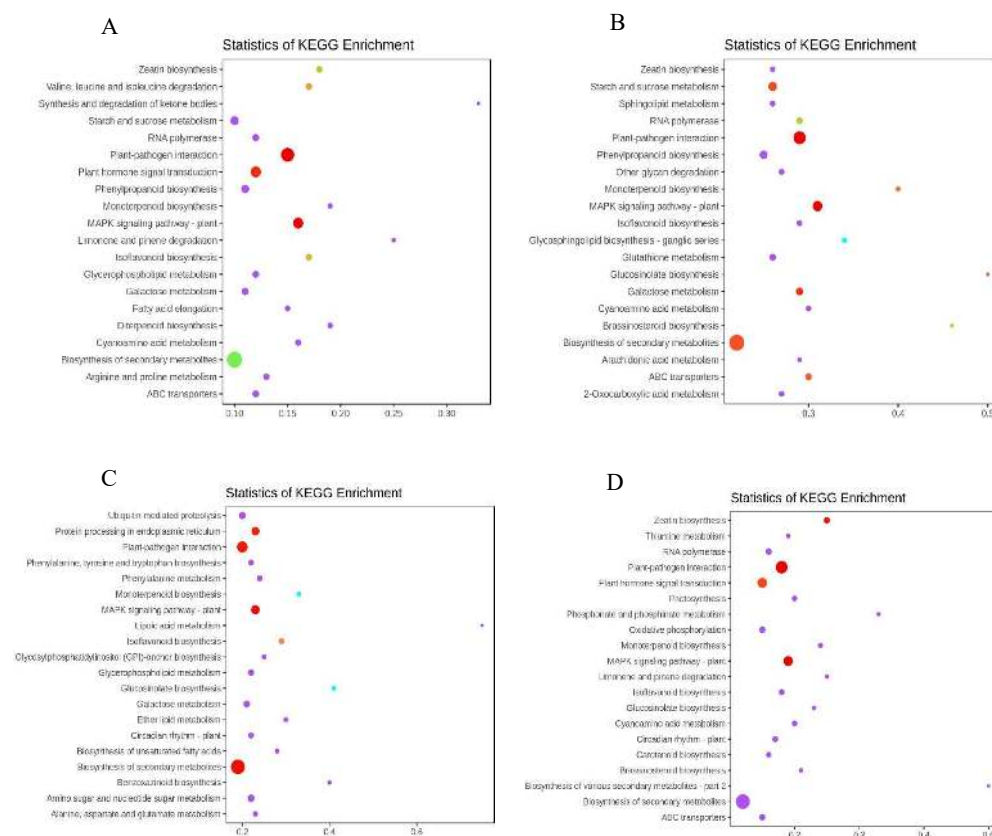


Figure 5. KEGG enrichment analysis of DEGs in *A. pseudosieboldianum* under freezing stress at different temperatures. KEGG pathway classification of DEGs of *A. pseudosieboldianum* under low-temperature stress at different temperatures and CK group control, respectively. The y-axis corresponds to the KEGG pathway, the x-axis indicates the number of DEGs enriched in a pathway between the enrichment rate. The color of the dot represents the q-value and the size of the dot represents the number of DEGs mapped to the reference pathway. A larger rich factor indicates a greater enrichment. A smaller q-value indicates a more significant enrichment. (A) is the KEGG enrichment analysis of DEGs between APCK (4 °C) and APA (−10 °C), (B) is the KEGG enrichment analysis of DEGs between APCK (4 °C) and APB (−20 °C), (C) is the KEGG enrichment analysis of DEGs between APCK (4 °C) and APC (−30 °C), (D) is the KEGG enrichment analysis of DEGs between APCK (4 °C) and APD (−40 °C).

2.5. Identification and Analysis of Genes Associated with the MAPK Pathway

The mitogen-activated protein kinases (MAPK) signaling pathway is one of the important pathways in the eukaryotic signaling network and is a key signaling pathway for cell proliferation, differentiation, apoptosis, and stress responses under normal and pathological conditions (Figure 6). As shown in Figure 6, genes on MAPK produced up-regulated or downregulated expression under freezing stress at different temperatures. Among them, upregulated genes produced up to 1.69-fold expression and downregulated genes produced up to 3.06-fold expression (Table S3).

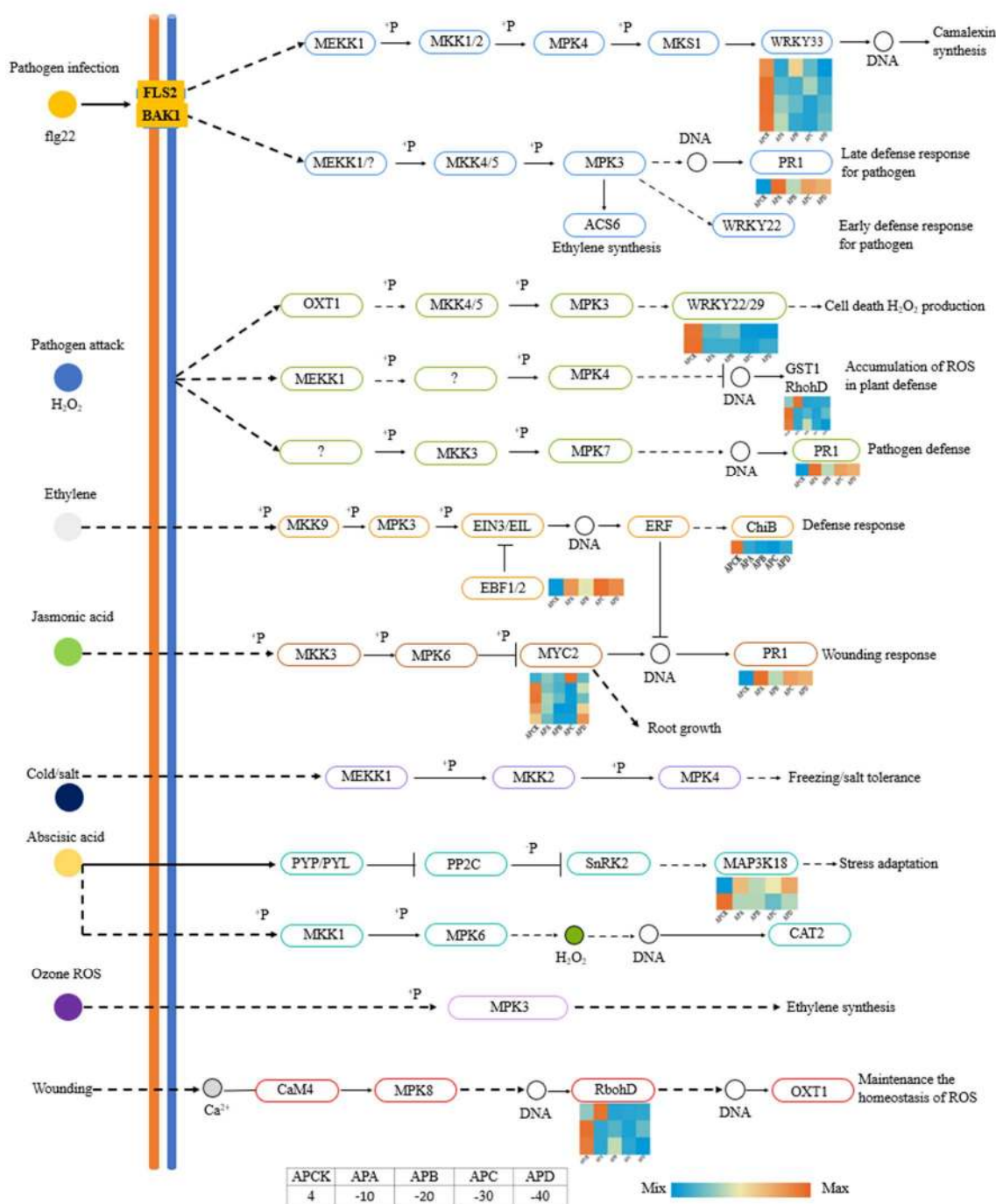


Figure 6. Expression of DEGs on MAPK pathway in *A. pseudosieboldianum* under freezing stress at different temperatures. The heatmap expression shows the differential gene expression of *A. pseudosieboldianum* under freezing stress at different temperatures compared to CK (4 °C).

2.6. Identification and Analysis of Genes Related to Sugar Metabolism

Sugars play a crucial role in the cold resistance of plants. In plant response to freezing stress, the accumulation of soluble sugars is usually detected. Soluble sugars can act as nutrients, osmoprotectants, and cryoprotectants to protect plant cells from damage caused by freezing stress. As shown in Figure 7, genes on the sugar metabolism pathway produced significant expression under freezing stress conditions at different temperatures (Table S4).

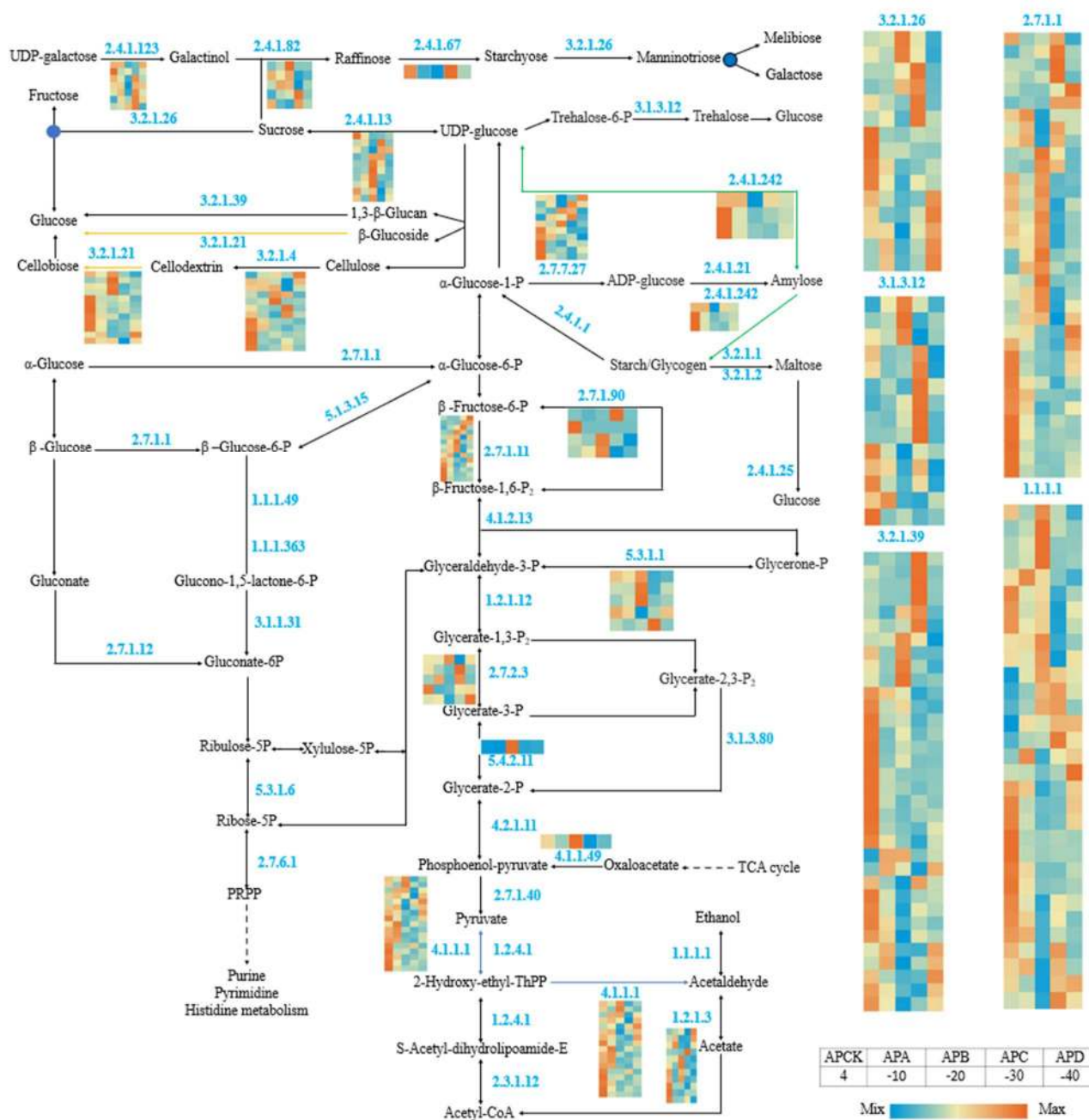


Figure 7. Expression of DEGs in the gluconeogenic pathway of *A. pseudosieboldianum* under freezing stress at different temperatures. The heat map shows the differential gene expression of *A. pseudosieboldianum* under freezing stress at different temperatures compared to CK (4 °C).

2.7. Key Transcription Factors Associated with Freezing Stress

In this study, we first determined all transcription factors and further analyzed the expression of key transcription factors (MYB, WRKY, AP2/ERF-ERF) (Figure 8) that are involved in freezing stress. The FPKM values of these transcription factors are visible in the Supplementary Materials (Table S5). In total, we found 115 MYB family members, 108 AP2/ERF-ERF family members, and 66 WRKY family members in all libraries (Figure 9).

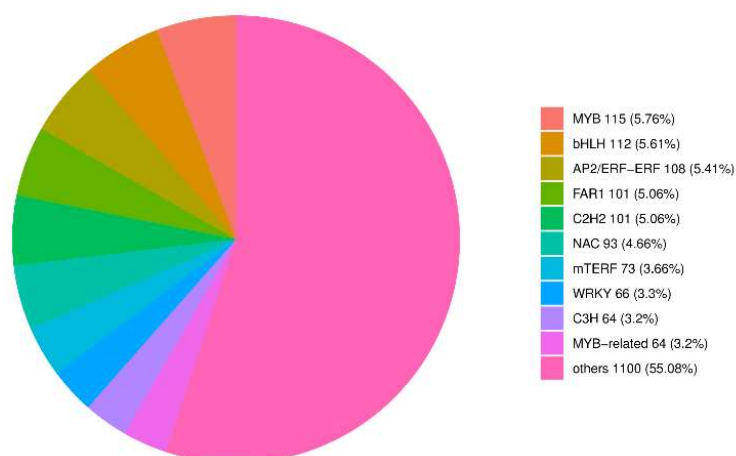


Figure 8. Transfer factors of *A. pseudosieboldianum* under freezing stress at different temperatures.

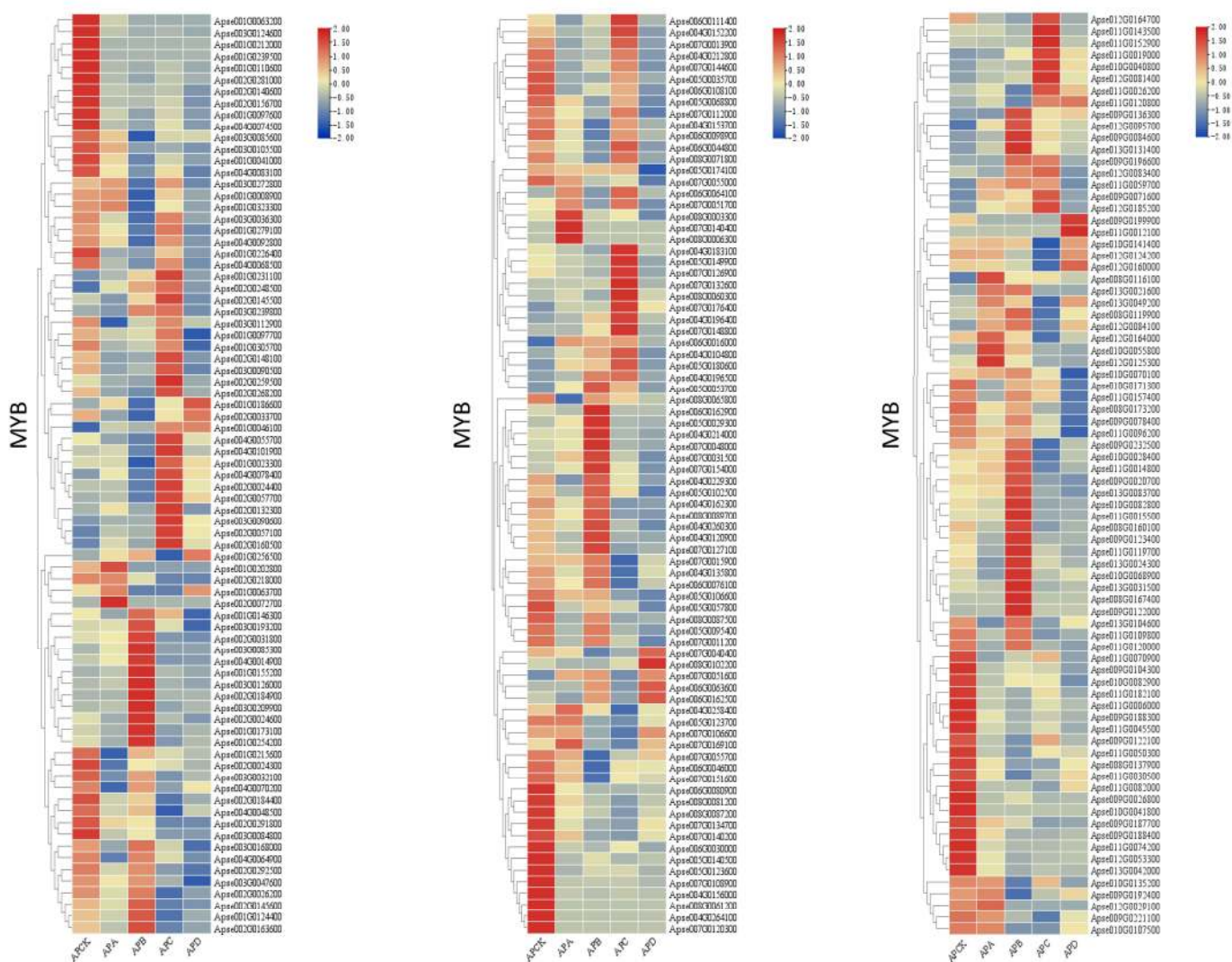


Figure 9. Cont.

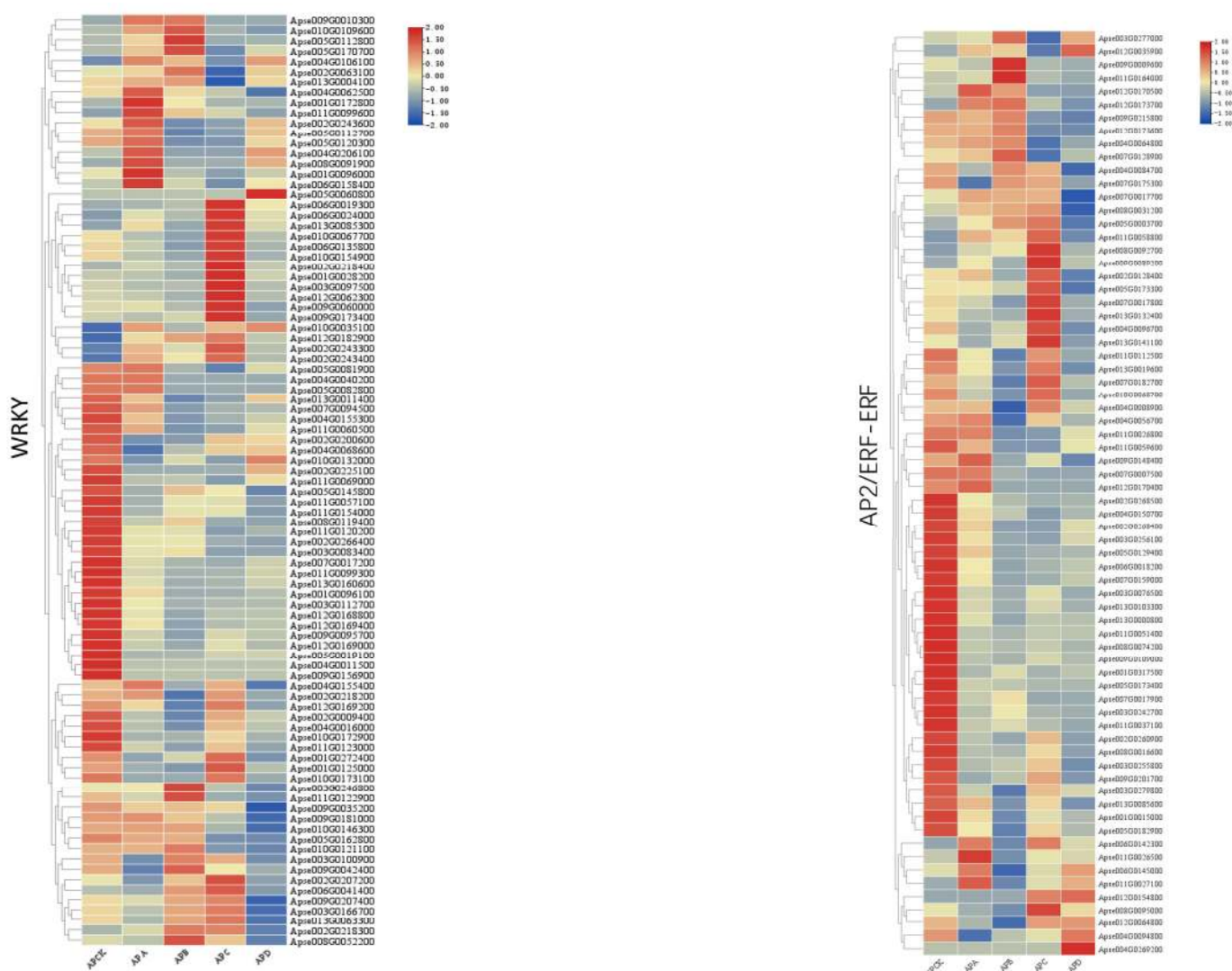


Figure 9. Expression of MYB, AP2/ERF, and WRKY genes. APCK: *A. pseudosieboldianum* treated at 4 °C; APA: *A. pseudosieboldianum* treated at −10 °C; APB: *A. pseudosieboldianum* treated at −20 °C; APC: *A. pseudosieboldianum* treated at −30 °C; APD: *A. pseudosieboldianum* treated at −40 °C.

3. Discussion

With the gradual improvement of living standards, the construction of an ecological environment is becoming more and more important. Foliage plants can not only be used as timber forests but also play an important role in urban ecological construction. Due to the influence of the relatively arid climate and low temperature in northern China, the distribution of colorful foliage plants in northern China is relatively small, and *A. pseudosieboldianum*, as an excellent colorful page plant in the north, is important to study in relation to its freezing stress. Meanwhile, due to the frequent occurrence of extreme weather in recent years, climate affects the development of many plants and even leads to death. The study of *A. pseudosieboldianum*'s response to abiotic stress also becomes very important. Multiple responses to freezing stress have been reported for many plant species, reflected in transcriptional or metabolic changes [37]. Recently, significant progress has been made in understanding the perception and transduction of cold signals in plants; however, we are still far from fully understanding the molecular mechanisms of cold signal perception and transduction in plants [38–40]. In our study, the genes responsible for resistance to freezing stress were identified by comparing the transcriptome profiles of *A. pseudosieboldianum* under freezing stress at different temperatures. We performed a more

comprehensive analysis of the changes in important metabolic pathways and transcription factors in *A. pseudosieboldianum*. In addition, our study provides reference value for other woody plants and provides theoretical support and facilities for future development of breeding to improve the breeding of cold-tolerant species of *A. pseudosieboldianum*. In this study, the freezing stress of *A. pseudosieboldianum* was studied from three aspects: hormone, enzyme activity and transcriptome (Figure 10).

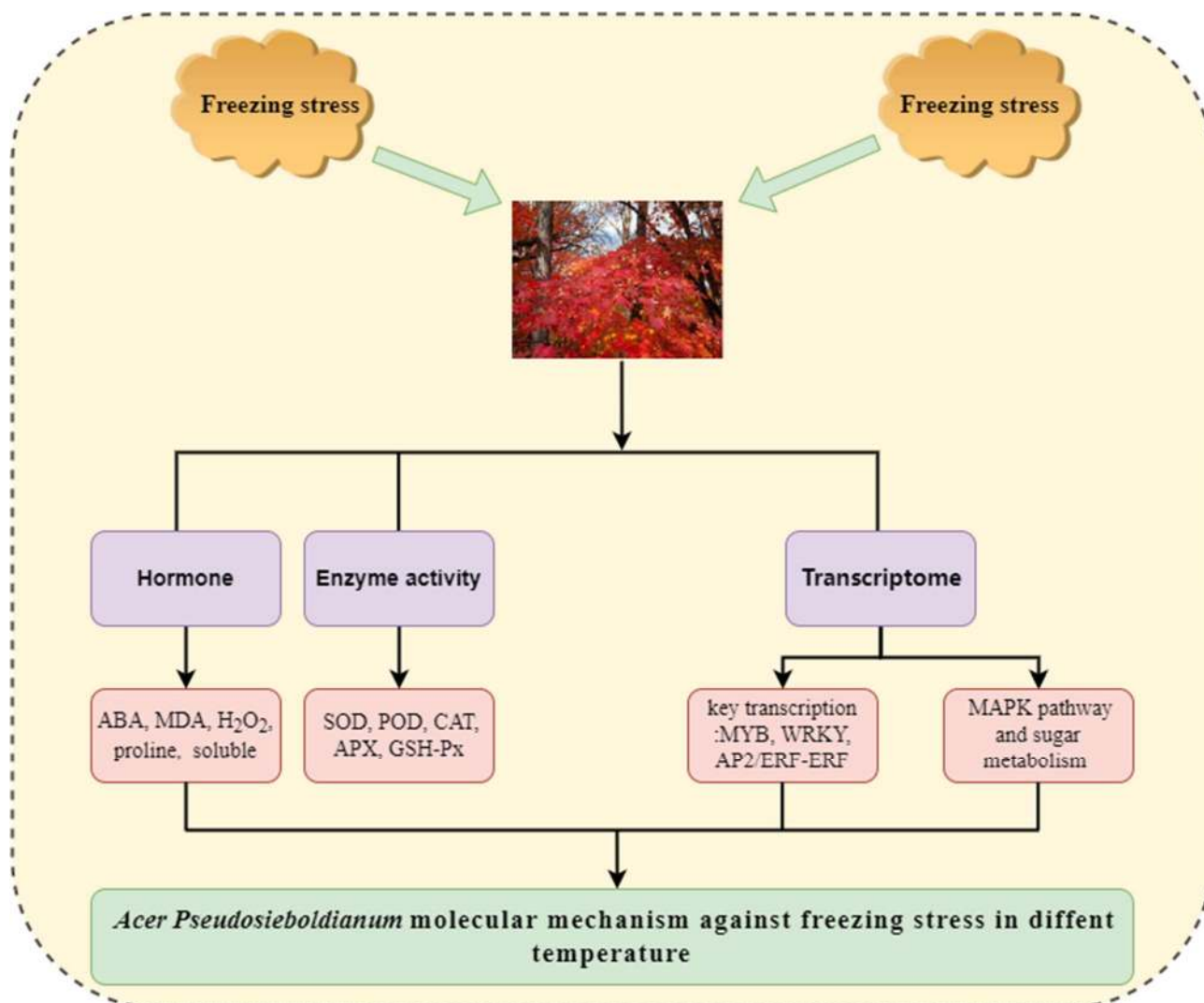


Figure 10. *A. pseudosieboldianum* freezing stress summary figure.

3.1. Identification of DEGs of *A. pseudosieboldianum* under Freezing Stress at Different Temperatures

Under freezing stress at different temperatures, 2505, 6021, 5125, and 3191 DEGs were identified, respectively, by comparison with the control (4 °C). Most of the important genes produced downregulation compared to the control. This result was consistent with the regulation of freezing stress in potatoes studied by Kou et al. [41]; however, the study of Niu et al. [42] on *Prunus persica* cold-tolerance resulted in a greater number of upregulated genes than downregulated genes for different levels of freezing stress, compared with the control group.

In addition, the GO classification of *A. pseudosieboldianum* genes under different temperature treatments was compared, and the functional categories were enriched, mainly in “metabolic processes”, “cellular processes”, and “response to stimuli”, and the KEGG pathway analysis showed that the main enriched pathways included phytohormone signaling,

metabolic pathways (Tables S6–S11), and ribosomal and secondary metabolic pathways. The comprehensive analysis of data from *A. pseudosieboldianum* under different temperature treatments identified more important DEGs involved in freezing stress and contributed to further understanding of *A. pseudosieboldianum*. In key gene families, many genes were significantly upregulated at CK (4 °C), −20 °C, −30 °C. However, genes were not significantly upregulated at −40 °C, which may be due to the fact that gene families such as MYB have begun to regulate under low temperature stress. The regulation is still obvious at −20 °C and −30 °C, and it is no longer regulated at −40 °C, which may be related to the extreme temperature of gene regulation of *A. pseudosieboldianum* in response to freezing stress. Meanwhile, the upregulation and downregulation changes of these three key gene families may be related to abiotic stress in *A. pseudosieboldianum*.

3.2. Genes Involved in the Sugar Metabolism Pathway under Freezing Stress

Sugars play a crucial role in the cold tolerance of plants. In plant response to cold freezing stress, the accumulation of soluble sugars is usually detected. Soluble sugars can act as nutrients, osmoprotectants, and cryoprotectants to protect plant cells from cold freezing stress damage. Sugars can also act as signaling molecules, similar to hormone, involved in plant growth, development, and various stress responses [43–45]. Therefore, several genes related to sugar metabolism were explored in this study. Sucrose, cottonseed sugar, glucose, and fructose accumulate under cold freezing stress and are involved in plant cold resistance [43,46]. Sucrose synthase and sucrose phosphate synthase are two important enzymes involved in sucrose metabolism and biosynthesis, respectively [47]. Here, the induction of 10 genes encoding sucrose synthases may point to the degradation of sucrose to UDP-glucose and fructose. This result shows the induction of sucrose synthase gene expression under freezing stress. Sucrose content and cold tolerance are positively correlated in woody plants [44,48]. Meanwhile, as shown in Figure 6, a total of 524 coding genes were involved in sugar metabolism, including 2.4.1.123 (inositol 3- α -galactosyltransferase), 2.4.1.67 (stachyose synthetase), 3.2.1.26 (Alkaline/neutral invertase), and 2.4.1.82 (raffinose synthase) in *A. pseudosieboldianum* at −20 °C and −30 °C. This demonstrated that sugar metabolism in *A. pseudosieboldianum* was about to be affected by freezing stress. This may be because *A. pseudosieboldianum* needs sugar to provide energy in the process of resisting freezing stress, so under the conditions of −20 °C and −30 °C, the genes are significantly upregulated, but under the freezing environment of −40 °C, the gene for *A. pseudosieboldianum* was no longer upregulated, probably because the enzyme had been inactivated in the plant.

3.3. Related Genes on MAPK Signaling Pathway under Freezing Stress

When growth and developmental or environmental signals occur and are delivered to effector cells, receptors located on the cell membrane, as well as intracellular signaling components, are required to deliver the signals to target sites in the cytoplasm or nucleus to elicit a cellular response. MAPK is an important component of the signaling pathway in eukaryotes, consisting of three cascades: MEKK (MAPKKK, MAP3K), MEK (MPKK, MAP2K), and MPK (MAPK), which are activated by phosphorylation, then, in turn, phosphorylate the downstream components to pass the signal on. When cells receive external signals, the receptors on the cell membrane are activated first, then the MEKK→MEK→MPK cascade is activated sequentially, and finally, MPK activates downstream targets, such as enzymes and transcription factors, causing a cellular response (Figures 1 and 2). MAPK is involved in a wide range of physiological processes in plants, including growth and development and plant response to biotic and abiotic stresses [49,50].

From Figure 5, we can see that *A. pseudosieboldianum* showed significant gene expression in pathogen defense (PR1) and stress adaptation (MAP3K18) under the effect of freezing stress. The highest gene expression was observed in stress adaptation (MAP3K18) and pathogen defense (PR1) under −40 °C treatment. It can be seen from the above that the

MAPK pathway still plays a significant role in responding to extremely low temperature environments, and it is upregulated at $-20\text{ }^{\circ}\text{C}$, $-30\text{ }^{\circ}\text{C}$ and $-40\text{ }^{\circ}\text{C}$.

3.4. Transcriptional Regulation-Related Genes under Freezing Stress

Transcription factors play important functions in abiotic stresses in plants (Tables S12 and S13). It has been shown that in Arabidopsis, most cold-regulated transcription factor genes are impaired in the plant response to cold stress [51–53]. It has also been shown that many transcription factors produce important roles in plant cold stress [54,55]. For example, plants induce the expression of cold-regulated genes (COR) to withstand freezing stress. COR genes in Arabidopsis include COR, LTI low-temperature induced (LTI) genes, responsive to desiccation (RD) genes, and early dehydration-inducible (ERD) genes, which encode key enzymes for the synthesis of osmoregulatory substances that increase tolerance by CBFs/DREB1 recognizing and binding the CRT/DRE cis-acting element containing the conserved sequence of CCGAC on the COR gene promoter to regulate the expression of COR genes. When plants are exposed to freezing stress, CBFs genes are rapidly induced to be expressed, and subsequently, COR gene expression is activated. For example, the AP2/ERF factors RAP2.1 and RAP2.6 and the C2H2-type zinc finger STZ/ZAT10 belong to the CBF regulators [56,57]. Many MYB-type transcription factors, such as AtMYB85 [58] and AtMYB88 [59], increase chilling tolerance in apple through CBF-dependent and CBF-independent pathways. In this study, we analyzed the expression patterns of the MYB, WRKY, and AP2/ERF-ERF gene families and found that different members play different roles under different levels of freezing stress. For example, in the WRKY family, different members of the family were involved in the response to low temperature.

Among these gene families, *Apse001G0028200* and *Apse002G0128400* showed significant changes in freezing stress treatment at $-30\text{ }^{\circ}\text{C}$; *Apse011G0120800* showed significant changes in freezing stress treatment at $-30\text{ }^{\circ}\text{C}$ and $-40\text{ }^{\circ}\text{C}$. These differences suggest that *A. pseudosieboldianum* has significant changes in transcription factors under different levels of freezing stress, thus verifying that these gene families may have regulatory mechanisms in the freezing stress of *A. pseudosieboldianum*. From the experimental results, it can be concluded that these differential genes may be related to the response of *A. pseudosieboldianum* to freezing stress. Among them, the *Apse011G0120800* gene may be related to the response to extremely low temperature. Many genes are not upregulated at $-40\text{ }^{\circ}\text{C}$, which may be caused by the extreme low temperature environment, in which the freezing resistance gene of *A. pseudosieboldianum* could no longer be expressed.

3.5. Changes in Physiological Indicators of *A. pseudosieboldianum* under Different Temperatures of Freezing Stress

The mechanisms that respond to low temperature stress in plants are complex and diverse. Once the low temperature signal is received, defense mechanisms are turned on, including physical structural adaptation (e.g., changes in membrane conformation), intercellular regulation of osmotic substances (e.g., Ca^{2+} , soluble sugars, proline, betaine) and scavenging of reactive oxygen species by protective enzyme systems (POD, SOD, others) [60]. In this study, the physiological responses of *A. pseudosieboldianum* under different levels of freezing stress were observed by measuring the activities of five enzymes, SOD, POD, CAT, APX and GSH-Px, and the contents of five hormones and metabolites, ABA, MDA, H_2O_2 , proline and soluble sugar, at different temperatures of freezing stress. The physiological responses of *A. pseudosieboldianum* under different temperatures of freezing stress were observed. ABA is a gibberellin and cytokinin inhibitor, which is related to leaf senescence and fruit abscission. Freezing stress can induce the accumulation of ABA, and ABA can induce the production of new proteins, which are involved in regulating the content of polyamines and soluble carbohydrates, increasing the activity of antioxidant enzymes, and enhancing the cold tolerance of plants [61–63]. From Figure 7, we can see that the activities of POD, CAT, and APX gradually increased until $-30\text{ }^{\circ}\text{C}$ and reached

the highest at $-30\text{ }^{\circ}\text{C}$, while SOD had the highest activity at $-20\text{ }^{\circ}\text{C}$. The hormone contents of ABA, MDA, H_2O_2 , proline, and soluble sugar gradually increased until $-30\text{ }^{\circ}\text{C}$, reached the highest at $-30\text{ }^{\circ}\text{C}$, and decreased immediately thereafter. From the graph, we speculate that in $-40\text{ }^{\circ}\text{C}$ the enzyme activity was reduced due to the reduced activity of *A. pseudosieboldianum* samples and the hormones could not continue to accumulate. As can be seen from the slope of the line graph, in response to freezing stress, the activities of POD, CAT and APX increased significantly in CK ($4\text{ }^{\circ}\text{C}$) $\sim -20\text{ }^{\circ}\text{C}$, and responded rapidly to freezing stress, the activity of SOD increased significantly at CK ($4\text{ }^{\circ}\text{C}$) $\sim -10\text{ }^{\circ}\text{C}$, and responded rapidly to freezing stress; the activity decreased significantly at $-40\text{ }^{\circ}\text{C}$, possibly due to the inactivation of the enzyme due to extreme low temperature. In endogenous hormones, ABA, MDA, H_2O_2 , proline, and soluble sugar accumulates rapidly in plants at CK ($4\text{ }^{\circ}\text{C}$) $\sim -30\text{ }^{\circ}\text{C}$. In this study, at $-40\text{ }^{\circ}\text{C}$, it dropped significantly again, which may be because the genes that control endogenous hormones in *A. pseudosieboldianum* are no longer upregulated at $-40\text{ }^{\circ}\text{C}$.

4. Materials and Methods

4.1. Plant Materials

Four-year-old *A. pseudosieboldianum* seedlings were collected from the greenhouse of Northeast Forestry University. The collection was made on 19 November 2021, at a temperature ranging from $8\text{ }^{\circ}\text{C}$ to $4\text{ }^{\circ}\text{C}$. Forty pots of current-year branches of four-year-old *A. pseudosieboldianum* seedlings of approximately the same growth were taken. The collected branches were left at $4\text{ }^{\circ}\text{C}$ and $0\text{ }^{\circ}\text{C}$ for 10 h and used as a control (APCK), while the other branches were treated and kept at freezing temperatures for 10 h: $-10\text{ }^{\circ}\text{C}$ (APA), $-20\text{ }^{\circ}\text{C}$ (APB), $-30\text{ }^{\circ}\text{C}$ (APC), and $-40\text{ }^{\circ}\text{C}$ (APD). In each treatment, 20 branches were divided into four groups, three as transcriptional samples and one as a physiological and biochemical sample. The xylem of the branches was then collected and snap-frozen in liquid nitrogen.

4.2. Determination of Physiological Indicators

In this study, the hormone contents and enzyme activities of five enzymes were determined in five groups of *A. pseudosieboldianum* under freezing stress at different temperatures. ABA levels were determined by double antibody sandwich assay; MDA, soluble sugar, proline, and H_2O_2 were determined by double antibody one-step sandwich enzyme-linked immunosorbent assay. COD, SOD, CAT, APX, and GSH-Px were determined by double antibody one-step sandwich enzyme-linked immunosorbent assay.

4.3. RNA Library Construction and Sequencing

The starting RNA for library construction was total RNA, $\geq 1\text{ }\mu\text{g}$. The library was built using Illumina's NEBNext® UltraTM RNA Library Prep Kit. mRNA with polyA tails was enriched by Oligo(dT) beads, and the resulting mRNA was randomly interrupted with divalent cations in NEB fragmentation buffer. The fragmented mRNA was used as a template and random oligonucleotides were used as primers to synthesize the first strand of cDNA in the MMuLV reverse transcriptase system, followed by degradation of the RNA strand with RNaseH and synthesis of the second strand of cDNA with dNTPs in the DNA polymerase I system. The purified double-stranded cDNA was end-repaired, A-tailed, and sequenced, and the cDNA of approximately 200bp was screened with AMPure XP beads, PCR amplified, and the PCR products were purified again with AMPure XP beads to obtain the final library. After the library was constructed, it was initially quantified using a Qubit2.0 fluorometer, diluted to $1.5\text{ ng}/\mu\text{L}$, and then the insert size of the library was measured using an Agilent 2100 bioanalyzer. After the insert size met the expectation, qRT-PCR was performed to accurately quantify the effective library concentration (i.e., the effective library concentration above 2 nM) to ensure the quality of the library.

After passing the library check, the different libraries were pooled by effective concentration and the target downstream data volume, then sequenced by Illumina, and

150 bp paired-end reads were generated. The basic principle of sequencing is sequencing by synthesis. Four fluorescently labeled dNTPs, DNA polymerase, and splice primers were added to the sequencing flow cell for amplification. When each sequencing cluster extended the complementary strand, each fluorescently labeled dNTP was added to release the corresponding fluorescence, and the sequencer captured the fluorescence signal and converted the light signal into sequencing peaks through computer software to obtain sequence information about the fragment to be sequenced.

4.4. Comparison of Transcriptome Data with the Reference Genome

Sequenced fragments are randomly interrupted by mRNA. To determine which genes these fragments are transcribed from, clean reads from quality control need to be matched to the reference genome. The clean reads are sequenced against the reference genome using HISAT2 (https://download.cncb.ac.cn/gwh/Plants/Acer_pseudosieboldianum_Apse_genome_GWHBECT00000000/GWHBECT00000000/GWHBECT0000.genome.fasta.gz, accessed on 6 October 2022) [64,65] to obtain information on the position of the reference genome or gene, as well as information on the sequence characteristics specific to the sequenced sample. The algorithm of HISAT2 [65,66] is divided into three main parts: (1) whole segment alignment of sequenced sequences to single exons of the genome; (2) segment alignment of sequenced sequences to two exons of the genome; and (3) segment alignment of sequenced sequences to three or more exons of the genome.

4.5. Differential Gene Screening

DESeq2 [67,68] was used to analyze genes expressed differentially among the sample groups, and the differentially expressed genes between the two biological conditions were obtained. After inputting the unstandardized reads counting data of genes, the multiple hypothesis test and correction of the hypothesis test probability (P value) was carried out by the Benjamini–Hochberg method, and the error detection rate (false discovery rate, FDR) was obtained. The screening condition of differential genes was $|\log_2\text{Fold Change}| \geq 1$, and $\text{FDR} < 0.05$.

4.6. RT-qPCR Validation

RNA-seq data were validated by RT-qPCR analysis (Figure S1). Amplification was performed using a 7500 Fast Real-Time PCR System and SYBRP remix EXTaQ kit (Takara, Kyoto, Japan). The reaction protocol was as follows: 95 °C for 30 s, 45 cycles, 95 °C for 5 s, 59 °C for 15 s, 72 °C for 20 s, and 72 °C for 7 min. Table S1 lists all the specific primers used. Three technical replicates were used for each sample (Tables S14–S17). ACTIN (accession number: AY261523.1) was used as a quantitative control to determine the relative expression values according to the $2^{-\Delta\Delta\text{ct}}$ algorithm [69].

5. Conclusions

In this study, transcriptome sequencing was first used to analyze *A. pseudosieboldianum* under different temperature freezing stresses at different temperatures. We considered the important genes involved in signal transduction, transcription, and regulation under freezing stress at different temperatures.

There was differential expression in *A. pseudosieboldianum*. Some different members of the MYB, WRKY, and AP2/ERF-ERF gene families were significantly upregulated/downregulated under different-temperature freezing stress. In addition, some DEGs involved in glucose metabolism, such as *Apse010G0038900* and *Apse001G0122200*, were significantly upregulated during freezing at $-30\text{ }^{\circ}\text{C}$, which further emphasized the importance of glucose metabolism in plant freezing tolerance.

As an important part of the signal transduction pathway in eukaryotes, MAPK also played an important role in freezing stress. Among the genes involved in MAPK signaling pathway, the genes treated at $-40\text{ }^{\circ}\text{C}$ were significantly upregulated in MAP3K18 and PR1.

In the physiological indicators of hormone and enzyme activity, *A. pseudosieboldianum* with the decrease of treatment temperature, enzyme activity, and hormone accumulation increased gradually. Under the freezing stress of -30°C , the activities of POD, CAT, and APX were the highest, and the accumulation of ABA, MDA, H_2O_2 , proline, and soluble sugar was the highest. When the temperature was lower than -30°C , the enzyme activity and hormone level decreased gradually, which was speculated to be caused by the decrease in the main body activity of *A. pseudosieboldianum* sample.

The results of this study can explain the molecular mechanism and play an important role in the further study of the cold tolerance of plants. The important DEGs found in this study may be helpful in further studying the freezing tolerance mechanism of *A. pseudosieboldianum* and other woody plants.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms232314676/s1>.

Author Contributions: Data curation, Z.H. and S.Z.; formal analysis, S.Z.; investigation, H.L. and Q.Z.; software, H.L. and X.L.; writing, Z.H.; funding acquisition, X.Z.; project administration, X.Z.; collection of test samples, X.X., Y.C., L.W. and S.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Heilongjiang Province Applied Technology Research and Development Plan Project (No. GA20B402), the Fundamental Research Funds for the Central Universities (Northeast Forestry University) (No. 2572020DR01), and the Heilongjiang Touyan Innovation Team Program (Tree Genetics and Breeding Innovation Team).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The *A. pseudosieboldianum* raw genome sequencing data are available from the NCBI under project ID PRJCA006356. *A. pseudosieboldianum* A. *pseudosieboldianum* Transcriptome or Gene expression in freezing stress from the NCBI under project ID PRJNA894936.

Acknowledgments: Thanks to the members of the State Key Laboratory of Tree Genetics and Breeding of Northeast Forestry University, College of Forestry and Grassland of Jilin Agricultural University, for their assistance during laboratory works and for fruitful discussions. Thanks also for the support of the Funds for the Central Universities project. We are grateful to/thank Wuhan Metware Biotechnology Co., Ltd., for assisting in the sequencing and/or bioinformatics analysis.

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

References

1. Yang, J.; Muhammad, W.H.; Tao, L.; Zhang, R.; Yun, Q.; Peter, H.; Zhiling, D.; Luo, G.; Guo, H.; Ma, Y. De novo genome assembly of the endangered *Acer yangbiense*, a plant species with extremely small populations endemic to Yunnan Province, China. *GigaScience* **2019**, *7*, giz085. [CrossRef] [PubMed]
2. Grossman, J.J. Evidence of Constrained Divergence and Conservatism in Climatic Niches of the Temperate Maples (*Acer* L.). *Forests* **2021**, *12*, 535. [CrossRef]
3. Gelderen, D.; Jong, P.; Oterdoom, H.J. *Maples of the World*; Timber Press: Portland, OR, USA, 1994.
4. Ma, Q.; Sun, T.; Li, S.; Wen, J.; Zhu, L.; Yin, T.; Yan, K.; Xu, X.; Li, S.; Mao, J.; et al. The *Acer truncatum* genome provides insights into nervonic acid biosynthesis. *Plant J.* **2020**, *104*, 662–678. [CrossRef]
5. Zhu, Z.; Lin, L. *Studies on Germplasm Resources and Cultivation Techniques of Maple*; Science Press: Shanghai, China, 2014.
6. Li, X.; Li, T.; Hong, X.Y.; Liu, J.J.; Liu, G.P. *Acer truncatum* Seed Oil Alleviates Learning and Memory Impairments of Aging Mice. *Front. Cell Dev. Biol.* **2021**, *9*, 680386. [CrossRef]
7. Aba, B.; Ddhib, C.; Jssb, D. Genome-wide supermatrix analyses of maples (*Acer*, Sapindaceae) reveal recurring inter-continental migration, mass extinction, and rapid lineage divergence. *Genomics* **2021**, *113*, 681–692.
8. Rong, H.; Xu, L.A. Study reviews of *Acer rubrum* L. introduction and application. *J. Jiangsu For. Sci. Technol.* **2020**, *47*, 39–44.
9. Quambusch, M.; Bucker, C.; Haag, V.; Meier-Dinkel, A.; Liesebach, H. Growth performance and wood structure of wavy grain sycamore maple (*Acer pseudoplatanus* L.) in a progeny trial. *Ann. For. Sci.* **2021**, *78*, 15. [CrossRef]
10. Dong, H.; Xu, B.; Ji, K. Comparative transcriptome analysis of genes involved in response to thermal stress and leaf colour change of *Acer palmatum*. *Sci. Hortic.* **2019**, *255*, 77–85. [CrossRef]

11. He, X.; Li, D.-Z.; Tian, B. Diversity in seed oil content and fatty acid composition in *Acer* species with potential as sources of nervonic acid. *Plant Divers.* **2021**, *43*, 86–92. [\[CrossRef\]](#)
12. Zhu, J.; Cheng, H.-M.; Zhu, Y.-P.; Cao, W.; Chen, B.; Tian, X.-J. Geographic variations in leaf shape of *Acer ginnala* (Aceraceae). *Plant Syst. Evol.* **2015**, *301*, 1017–1028. [\[CrossRef\]](#)
13. Couturier, E.; Brunel, N.; Douady, S.; Nakayama, N. Abaxial growth and steric constraints guide leaf folding and shape in *Acer pseudoplatanus*. *Am. J. Bot.* **2012**, *99*, 1289–1299. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Royer, D.L.; Meyerson, L.A.; Robertson, K.M.; Adams, J.M. Phenotypic plasticity of leaf shape along a temperature gradient in *Acer rubrum*. *PLoS ONE* **2009**, *4*, e7653. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Wahlsteen, E. SSR markers distinguish critically endangered *Acer campestre* populations from cryptic invading gene pools. *Willdenowia* **2021**, *51*, 115–125. [\[CrossRef\]](#)
16. Li, X.; Li, M.; Hou, L.; Zhang, Z.; Pang, X.; Li, Y. De Novo Transcriptome Assembly and Population Genetic Analyses for an Endangered Chinese Endemic *Acer miaotaiense* (Aceraceae). *Genes* **2018**, *9*, 378. [\[CrossRef\]](#)
17. Kim, H.J.; Kim, G.T. Flowering, Fruiting, Seed Fall and Seed Viability of *Acer pseudosieboldianum* in Mt. Jungwang, Gangwondo. *J. Korean Soc. For. Sci.* **2016**, *105*, 42–47. [\[CrossRef\]](#)
18. Gao, Y.-F.; Zhao, D.-H.; Zhang, J.-Q.; Chen, J.-S.; Li, J.-L.; Weng, Z.; Rong, L.-P. De novo transcriptome sequencing and anthocyanin metabolite analysis reveals leaf color of *Acer pseudosieboldianum* in autumn. *BMC Genom.* **2021**, *22*, 383. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Zhu, J.-K. Abiotic stress signaling and responses in plants. *Cell* **2016**, *167*, 313–324. [\[CrossRef\]](#)
20. Guo, X.; Liu, D.; Chong, K. Cold signaling in plants: Insights into mechanisms and regulation. *J. Integr. Plant Biol.* **2018**, *60*, 745–756. [\[CrossRef\]](#)
21. Jan, N.; Andrabi, K.I. Cold resistance in plants: A mystery unresolved. *Electron. J. Biotechnol.* **2009**, *12*, 14–15. [\[CrossRef\]](#)
22. Jia, Y.; Ding, Y.; Shi, Y.; Zhang, X.; Gong, Z.; Yang, S. The cbfs triple mutants reveal the essential functions of CBFs in cold acclimation and allow the definition of CBF regulons in *Arabidopsis*. *New Phytol.* **2016**, *212*, 345–353. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Chinnusamy, V.; Zhu, J.; Zhu, J.-K. Cold stress regulation of gene expression in plants. *Trends Plant Sci.* **2007**, *12*, 444–451. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Cai, K. Physiological effects of jasmonic acid on rice seedlings under low temperature stress. *Hubei Agric. Sci.* **2014**, *53*, 3512–3515. [\[CrossRef\]](#)
25. Sah, S.K.; Reddy, K.R.; Li, J. Absciscic acid and abiotic stress tolerance in crop plants. *Front. Plant Sci.* **2016**, *7*, 571. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Zhang, C. Research progress of transcriptomics in plant response to stress. *J. Biol.* **2017**, *34*, 86–90.
27. Zhou, H.; He, Y.; Zhu, Y.; Li, M.; Song, S.; Bo, W.; Li, Y.; Pang, X. Comparative transcriptome profiling reveals cold stress responsiveness in two contrasting Chinese jujube cultivars. *BMC Plant Biol.* **2020**, *20*, 240. [\[CrossRef\]](#)
28. Thomashow, M.F. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Biol.* **1999**, *50*, 571–599. [\[CrossRef\]](#)
29. Gilmour, S.J.; Fowler, S.G.; Thomashow, M.F. Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol. Biol.* **2004**, *54*, 767–781. [\[CrossRef\]](#)
30. Chinnusamy, V.; Schumaker, K.; Zhu, J.K. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *J. Exp. Bot.* **2004**, *55*, 225–236. [\[CrossRef\]](#)
31. Song, Y.; Chen, Q.; Ci, D.; Zhang, D. Transcriptome profiling reveals differential transcript abundance in response to chilling stress in *Populus simonii*. *Plant Cell Rep.* **2013**, *32*, 1407–1425. [\[CrossRef\]](#)
32. Yang, X.; Zhao, T.; Rao, P.; Gao, K.; Yang, X.; Chen, Z.; An, X. Transcriptome profiling of *Populus tomentosa* under cold stress. *Ind. Crops Prod.* **2019**, *135*, 283–293. [\[CrossRef\]](#)
33. Renaut, J.; Lutts, S.; Hoffmann, L.; Hausman, J.-F. Responses of poplar to chilling temperatures: Proteomic and physiological aspects. *Plant Biol.* **2004**, *7*, 81–90.
34. Sun, B.; Liang, M.; Yang, Y.; Xu, H. A primary analysis on cold resistance of *Acer pseudosieboldianum* (pax) kom in Heilongjiang Province. *Territ. Nat. Resour. Study* **2013**, *3*, 88–89.
35. Liu, C.; Tsuda, Y.; Shen, H.; Hu, L.; Saito, Y.; Ide, Y. Genetic structure and hierarchical population divergence history of *Acer mono* var. *mono* in South and Northeast China. *PLoS ONE* **2014**, *9*, e87187.
36. Ashburner, M.; Ball, C.A.; Blake, J.A.; Botstein, D.; Butler, H.; Cherry, J.M.; Davis, A.P.; Dolinski, K.; Dwight, S.S.; Eppig, J.T. Gene ontology: Tool for the unification of biology. *Nat. Genet.* **2000**, *25*, 25–29. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Shi, Y.; Ding, Y.; Yang, S. Molecular regulation of CBF signaling in cold acclimation. *Trends Plant Sci.* **2018**, *23*, 623–637. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Zhang, Z.; Li, J.; Li, F.; Liu, H.; Yang, W.; Chong, K.; Xu, Y. OsMAPK3 phosphorylates OsbHLH002/OsICE1 and inhibits its ubiquitination to activate OsTPP1 and enhances rice chilling tolerance. *Dev. Cell* **2017**, *43*, 731–743.e735. [\[CrossRef\]](#)
39. Pang, T.; Ye, C.-Y.; Xia, X.; Yin, W. De novo sequencing and transcriptome analysis of the desert shrub, *Ammopiptanthus mongolicus*, during cold acclimation using Illumina/Solexa. *BMC Genom.* **2013**, *14*, 488. [\[CrossRef\]](#)
40. Hong, J.H.; Savina, M.; Du, J.; Devendran, A.; Ramakanth, K.K.; Tian, X.; Sim, W.S.; Mironova, V.V.; Xu, J. A sacrifice-for-survival mechanism protects root stem cell niche from chilling stress. *Cell* **2017**, *170*, 102–113.e114. [\[CrossRef\]](#)
41. Kou, S.; Chen, Y.; Liu, T.; Liu, S.; Fang, Z.; Li, X.; Cai, X.; Song, B. Crosstalk of Putrescine Synthetic Pathway with Absciscic Acid Signaling Pathway in Cold Tolerance of Potato. *Environ. Exp. Bot.* **2022**, *204*, 105085. [\[CrossRef\]](#)

42. Niu, R.; Zhao, X.; Wang, C.; Wang, F. Transcriptome profiling of *Prunus persica* branches reveals candidate genes potentially involved in freezing tolerance. *Sci. Hortic.* **2020**, *259*, 108775. [\[CrossRef\]](#)
43. Ma, Y.; Zhang, Y.; Lu, J.; Shao, H. Roles of plant soluble sugars and their responses to plant cold stress. *Afr. J. Biotechnol.* **2009**, *8*, 2004–2010. [\[CrossRef\]](#)
44. Welling, A.; Palva, E.T. Molecular control of cold acclimation in trees. *Physiol. Plant.* **2006**, *127*, 167–181. [\[CrossRef\]](#)
45. Rütten, D.; Santarius, K.A. Relationship between frost tolerance and sugar concentration of various bryophytes in summer and winter. *Oecologia* **1992**, *91*, 260–265. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Anchordoguy, T.J.; Rudolph, A.S.; Carpenter, J.F.; Crowe, J.H. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiology* **1987**, *24*, 324–331. [\[CrossRef\]](#)
47. Winter, H.; Huber, S.C. Regulation of sucrose metabolism in higher plants: Localization and regulation of activity of key enzymes. *Crit. Rev. Plant Sci.* **2000**, *19*, 31–67. [\[CrossRef\]](#)
48. Sauter, J.J.; Wisniewski, M.; Witt, W. Interrelationships between ultrastructure, sugar levels, and frost hardiness of ray parenchyma cells during frost acclimation and deacclimation in poplar (*Populus × canadensis* Moench ‘robusta’) Wood. *J. Plant Physiol.* **1996**, *149*, 451–461. [\[CrossRef\]](#)
49. Du, X. H₂S signals and MAPK in *Arabidopsis thaliana* in Response to Drought and Cold Stress. Ph.D. Thesis, Shanxi University, Taiyuan, China, 2019.
50. Du, X.; Jin, Z.; Zhang, L.; Liu, X.; Yang, G.; Pei, Y. H₂S is involved in ABA-mediated stomatal movement through MPK4 to alleviate drought stress in *Arabidopsis thaliana*. *Plant Soil* **2019**, *435*, 295–307. [\[CrossRef\]](#)
51. Lee, B.-h.; Henderson, D.A.; Zhu, J.-K. The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. *Plant Cell* **2005**, *17*, 3155–3175. [\[CrossRef\]](#)
52. Lee, S.-C.; Lee, M.-Y.; Kim, S.-J.; Jun, S.-H.; An, G.; Kim, S.-R. Characterization of an abiotic stress-inducible dehydrin gene, OsDhn1, in rice (*Oryza sativa* L.). *Mol. Cells* **2005**, *19*, 212–218.
53. Zhao, C.; Wang, P.; Si, T.; Hsu, C.-C.; Wang, L.; Zayed, O.; Yu, Z.; Zhu, Y.; Dong, J.; Tao, W.A. MAP kinase cascades regulate the cold response by modulating ICE1 protein stability. *Dev. Cell* **2017**, *43*, 618–629.e615. [\[CrossRef\]](#)
54. An, J.-P.; Li, R.; Qu, F.-J.; You, C.-X.; Wang, X.-F.; Hao, Y.-J. An apple NAC transcription factor negatively regulates cold tolerance via CBF-dependent pathway. *J. Plant Physiol.* **2018**, *221*, 74–80. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Ding, Y.; Shi, Y.; Yang, S. Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. *New Phytol.* **2019**, *222*, 1690–1704. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Fowler, S.; Thomashow, M.F. *Arabidopsis* Transcriptome Profiling Indicates That Multiple Regulatory Pathways Are Activated during Cold Acclimation in Addition to the CBF Cold Response Pathway. *Plant Cell* **2002**, *14*, 1675–1690. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Vogel, J.T.; Zarka, D.G.; Van Buskirk, H.A.; Fowler, S.G.; Thomashow, M.F. Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. *Plant J.* **2005**, *41*, 195–211. [\[CrossRef\]](#)
58. Zhong, R.; Lee, C.; Zhou, J.; McCarthy, R.L.; Ye, Z.-H. A Battery of Transcription Factors Involved in the Regulation of Secondary Cell Wall Biosynthesis in *Arabidopsis*. *Plant Cell* **2008**, *20*, 2763–2782. [\[CrossRef\]](#)
59. Xie, Y.; Chen, P.; Yan, Y.; Bao, C.; Li, X.; Wang, L.; Shen, X.; Li, H.; Liu, X.; Niu, C.; et al. An atypical R2R3 MYB transcription factor increases cold hardiness by CBF-dependent and CBF-independent pathways in apple. *New Phytol.* **2018**, *218*, 201–218. [\[CrossRef\]](#)
60. Lee, D.H.; Lee, C.B. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: In gel enzyme activity assays. *Plant Sci.* **2000**, *159*, 75–85. [\[CrossRef\]](#)
61. Deng, F.F. Effects of exogenous ABA on proline accumulation and metabolic pathways in *Jatropha jatropha* seedlings under low temperature stress. *J. Plant Physiol.* **2015**, *51*, 221–226.
62. Guan, L.M.; Zhao, J.; Scandalios, J.G. Cis-elements and trans-factors that regulate expression of the maize Cat1 antioxidant gene in response to ABA and osmotic stress: H₂O₂ is the likely intermediary signaling molecule for the response. *Plant J.* **2000**, *22*, 87–95. [\[CrossRef\]](#)
63. Costa, R.; Lobato, A.; Silveira, J.; Laughinghouse HDJS; Turkey TRCo. ABA-mediated proline synthesis in cowpea leaves exposed to water deficiency and rehydration. *Turk. J. Agric. For.* **2011**, *35*, 309–317. [\[CrossRef\]](#)
64. Li, X.; Cai, K.; Han, Z.; Zhang, S.; Sun, A.; Xie, Y.; Han, R.; Guo, R.; Tigabu, M.; Sederoff, R.; et al. Chromosome-Level Genome Assembly for *Acer pseudosieboldianum* and Highlights to Mechanisms for Leaf Color and Shape Change. *Front. Plant Sci.* **2022**, *13*, 850054. [\[CrossRef\]](#)
65. Giorgi, F.M.; Ceraolo, C.; Mercatelli, D. The R Language: An Engine for Bioinformatics and Data Science. *Life* **2022**, *12*, 648. [\[CrossRef\]](#) [\[PubMed\]](#)
66. Kim, D.; Langmead, B.; Salzberg, S.L. HISAT: A fast spliced aligner with low memory requirements. *Nat. Methods* **2015**, *12*, 357–360. [\[CrossRef\]](#) [\[PubMed\]](#)
67. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [\[CrossRef\]](#)
68. Varet, H.; Brillet-Guéguen, L.; Coppée, J.-Y.; Dillies, M.-A. SARTools: A DESeq2-and EdgeR-based R pipeline for comprehensive differential analysis of RNA-Seq data. *PLoS ONE* **2016**, *11*, e0157022. [\[CrossRef\]](#) [\[PubMed\]](#)
69. Liao, W.; Ji, L.; Wang, J.; Chen, Z.; Ye, M.; Ma, H.; An, X. Identification of glutathione S-transferase genes responding to pathogen infestation in *Populus tomentosa*. *Funct. Integr. Genom.* **2014**, *14*, 517–529. [\[CrossRef\]](#)