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Comparative Transcriptome Analysis Revealed the New Role of Hormones in Flower Bud Differentiation of Peach Trees Under Different Chilling Hours

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Abstract: Peach bud differentiation is commercially significant for fruit production. Bud differentiation in peach production is closely linked to chilling requirements. This study investigates the mechanisms of flower bud differentiation in peach varieties L12 and N1 under varying chilling requirements by comparing paraffin sections, hormone content changes, and transcriptomes during four chilling hours and the same physiological stage. At 400 chilling hours, significant changes in flower bud differentiation were observed. During this period, the hormone levels of auxin and gibberellin reached their peak, while abscisic acid levels were at their lowest. This finding indicates that 400 chilling hours has a significant regulatory effect on flower bud development. Transcriptome analysis revealed that 4719 differentially expressed genes were identified in the flower bud differentiation of L12-4 and N1-4, and 2717 differentially expressed genes of L12-8 and N1-2, many of which are involved in IAA, GA, and ABA signal transduction pathways. In N1, the differentially expressed genes AUX/*IAA*, *SAUR*, and *DELLA* were significantly higher than in L12, whereas genes associated with the ABA synthesis pathway, such as *PYL2*, *PYL8*, and *SRK2A*, remained at the lowest level. This study provides a crucial molecular basis for understanding the regulation of plant hormones and their effects on flower bud development under varying chilling hours.

Keywords: chilling requirement; flower bud differentiation; hormone changes; the transcriptome

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1. Introduction

Peach (*Prunus persica*) is an important fruit tree resource cultivated worldwide. Its fruit is highly valued by consumers, and facile cultivation in temperate regions yields significant economic benefits [1]. The growth and development of peach trees are influenced by various internal and external factors, with the satisfaction of low-temperature requirements (chilling requirement) being a crucial factor in flower bud differentiation [2]. During the growth and development of peach buds, peach varieties with a chilling requirement of less than 450 h are called short chilling requirement varieties, and a 400 h chilling requirement is close to the threshold of short chilling requirement of peach, which plays an extremely important role in promoting the short chilling requirement of falling fruit trees and the breaking of rest [3]. During flower bud differentiation, the primordium of flowers or inflorescences is produced at the initial stage of the transition from vegetative to reproductive growth. The growth point protrudes, the tip flattens, and the flower bud is formed [4]. Subsequently, the inner cells of the flower bud transform into reproductive tissues, while the morphology of bud cells, tissue differentiation (formation of sepal primordium, differentiation of stamen and pistil), and physiological activities are adjusted [5].

Flower bud differentiation is a complex reaction process to the integration of signals from the external environment and internal factors [6]. The molecular mechanism of flower bud differentiation in peach trees has been well described [7]; however, the molecular

regulation of chilling requirements and flower bud differentiation in peach varieties remains underexplored. This is of great significance for cultivating peach varieties with different chilling requirements and analyzing the regulatory mechanism of dormancy [8].

In Arabidopsis thaliana, several genes have been identified to be involved in flower bud formation, including *FLOWERING LOCUS C* (*FLC*), *Suppressor of overexpression of CO 1*(*SOC1*), *LEAFY*(*LFY*), and *APETALA2* (*AP2*). The key genes *PpDAM6* and *H3K27me3* control the chilling requirement of peach blossom. These regulatory genes are mainly involved in plant hormones, vernalization, glucose metabolism, and other pathways, which can irreversibly induce plant flower bud differentiation [9]. Studies have shown that plant hormones regulate their synthesis and distribution in response to low temperature, thus regulating flower bud differentiation [10]. For example, at low temperatures, the content of abscisic acid (ABA) increases in response to cold conditions [11,12]. Abscisic acid (ABA) is also involved in flowering regulation. Transcription factors *ABA INSENSITIVE 4* (*ABI4*) and *ABI5*, which are involved in flower bud differentiation and can directly regulate the expression of *FLOWERING LOCUS C*, thus facilitating flowering regulation [13]. Concurrently, cytokinin levels change following low-temperature treatment, potentially affecting the flower bud development process [14]. Numerous studies indicate that when gibberellin (GA) is used as a promoter of flower bud differentiation, gibberellin content in Chinese cabbage significantly increases after low-temperature treatment (4 °C) and then decreases rapidly during vegetative growth, subsequently rising again during the flower bud budding stage, reaching a peak during flower bud differentiation [15]. Changes in the expression of GA synthesis-related genes (*GA20ox* and *GA3ox*) and signal transduction-related genes such as *Gibberellin Insensitive Dwarf1* (*GID1*) and *Gibberellin-regulated* (*DELLA*) under low-temperature conditions directly influence GA synthesis and activity [16]. Under low-temperature conditions, the expression of *GA20ox* and *GA3ox* may be regulated, reducing GA synthesis and promoting flower bud formation [17]. Other plant hormones, such as auxin (IAA) and the *AUX/IAA* gene family, as well as auxin synthesis and transport, are regulated under low-temperature conditions, impacting cell growth and differentiation. Changes in auxin levels can promote the formation of flower buds during the transition from vegetative to reproductive buds [18]. The *YUC* gene family is crucial in the IAA synthesis pathway, with its biosynthesis dependent on the tryptophan pathway, playing a vital role in regulating flower bud differentiation [19]. In studies of tomato flower organ loss, gene expression increases in the stamen and decreases in the pistil under low-temperature stress [20].

The identification and analysis of genes related to flower bud differentiation can reveal the physiological response mechanisms of various peach tree varieties under different chilling requirements. This not only aids in understanding the biological characteristics of peaches but also provides a scientific basis for breeding superior varieties that adapt to varying climatic conditions. This study aims to systematically investigate the characteristics of flower bud differentiation and the expression of hormone-related genes in peach varieties with high and low chilling requirements under four different chilling hours. By comparing the physiological responses of different varieties under varying chilling hours, this study identifies key regulatory genes and their mechanisms of action, providing theoretical support for cultivation management and the improvement of peach varieties.

2. Materials and Methods

2.1. Overview of the Test Site

The experimental site is situated in the Peach Variety Garden of the Gansu Academy of Agricultural Sciences in the Anning District of Lanzhou City, Gansu Province (103°41′ E, 36°6′ N), at an altitude of 1530 m. The annual average temperature is 9.6 °C, with an extreme minimum temperature of −25 °C. The frost-free period lasts for 196 days, and the annual effective accumulated temperature of ≥10 °C totals 3242 °C (the sum of the daily effective cumulative temperature we selected, daily effective accumulated temperature = the average temperature of the day (more than 15 °C) −10 °C). The average annual sunshine

duration is 2634 h, while the average annual precipitation measures 329 mm. Irrigation conditions are also available. The soil is characterized as yellow and spongy, with the topsoil containing 16.2 g·kg^{-1} of organic matter and a pH of 8.03.

2.2. Plant Materials

The experimental materials included "Longmi 12" (L12, with a chilling requirement of 700 h) and "Nanguitao 1" (N1, with a chilling requirement of 150 h) from the Tao Variety Garden of the Gansu Academy of Agricultural Sciences. "Longmi 12" (L12), which has a relatively high chilling requirement, served as the control group, while N1 functioned as the experimental group. The chilling hours were 0, 200, 400, and 800. Samples were collected at four chilling hours: 0, 200, 400, and 800. Flower bud samples, from which scales were removed, were frozen in liquid nitrogen and subsequently stored in a −80 °C refrigerator for paraffin section preparation and transcriptome sequencing analysis. Chilling hour calculation: After the peach orchard leaves fell and the daily minimum temperature was 7.2 °C, the daily temperature was recorded by a China Hangzhou Loggertech Co., Ltd., temperature and moisture recorder (Model L99-TWS-1). The recorder was placed 1.8 m above the ground, and it recorded every 10 min and then calculated the cumulative hours of low temperature in the range of 0 to 7.2 °C [21]. Sampling was carried out 1.8 m above the ground in the middle and upper part of the tree and according to the corresponding positions; and the sampling time was 3 November 2023, 17 November 2023, 4 December 2023, and 31 January 2024; and the chilling hours were 0, 200, 400, and 800 h, respectively.

2.3. Test Methods

Histological identification: The fixed tissue was prepared using an FAA fixative solution composed of 90% ethanol, 5% acetic acid, and 5% formaldehyde in a ratio of 90:5:5 mL per sample. Subsequently, the tissue underwent dehydration with varying concentrations of ethanol solutions, followed by clearing with xylene and embedding in paraffin wax (50% paraffin wax mixed with 50% xylene). The embedded tissue was then sectioned to a thickness of 5–8 microns using a rotary microtome. A single section was placed on a microscope slide, onto which a drop of RO water was added. The slide was subsequently positioned on a warming block to facilitate the melting of the wax and absorption of excess water. After drying at 37 °C, the slide underwent dewaxing and bleaching prior to staining with a 1% carmine solution. Finally, after allowing it to dry completely, the slide was examined under a microscope for observation of the anatomical structure of the flower bud.

Hormone-level analysis: We determined the contents of IAA, ABA, and GA in plant hormones by using the Shanghai COIBO BIO enzyme-linked immunoassay (ELISA) kit (96T), auxin ELISA test kit, GA ELISA test kit, and ABA ELISA test kit; 0.1 g flower bud samples were quickly frozen in liquid nitrogen and pre-cooled in a mortar; and then peach blossom buds were put in a mortar from liquid nitrogen, quickly ground, and placed in a pre-cooled centrifuge tube. Immediately after, 1 mL PBS buffer (pH 7.4) was added to its centrifuge tube for 30 s. The mixture was vortexed for 30 s to ensure complete mixing. The mixture was centrifuged at 3000 r·min^{-1} for 30 min to collect the supernatant, which was then packaged for the measurement of different hormone contents. The specific testing methods were followed according to the kit instructions. An enzyme-labeled Rayto RT-6100 was used for analysis, with each sample repeated 3 times.

Transcriptomics feature analysis: Total RNA was extracted from plants using the RNA prep Pure Plant Kit (Tiangen, Beijing, China). RNA purity and concentration were measured using the NanoDrop 2000 spectrophotometer (OD260/280 ratio between 1.8 and 2.0), and the Agilent 2100/LabChip GX detected RNA integrity accurately (the RIN value should be above 7.0). The Hieff NGS Ultima Dual-mode mRNA Library Prep Kit for Illumina (Yeasen Biotechnology, Shanghai, China) was utilized to generate the sequencing library. After purification and quality assessment, the library was sequenced on the Illumina NovaSeq platform. Further processing was conducted using the bioinformatics analysis

platform BMKCloud, and differential expression analysis was performed on the two groups using DESeq2. The KOBAS database and clusterProfiler 4.0 software were employed to analyze the enrichment of differentially expressed genes in the KEGG pathway (all gene sequences present in the gene expression analysis have been placed in the supplementary file All_Gene.longest_transcript).

2.4. Statistical Analysis

IBM SPSS Statistics 24 was used for data difference significance analysis (one-way ANOVA), and OriginPro 2022 software was used for charting.

3. Results

3.1. Determination of Flower Bud State Under Different Chilling Hours

The developmental state of buds is significantly influenced by environmental conditions. To assess the synchronization of flower bud development across varying chilling hours, internal morphological and developmental characteristics were examined using paraffin sections. We found that under the condition of short chilling requirement, the whole development process of flower buds was relatively slow, and the characteristics of flower bud development were not obvious on the whole (Figure 1). Under 0 chilling hours, the buds of L12 were smaller, the number of stamens was small, and the sepals and petals were in the initial state; under 800 chilling hours, the number of stamens increased, and the petal primordium differentiated, formed inside the calyx, and interformed with the calyx. The buds of N1 were small and the stamen quantity was small when the chilling hour requirement was 0 h. The buds began to grow at 200 h after chilling. At 400 h after chilling, the volume of the whole flower bud increased, the calyx primordium differentiated, the petal primordium formed inside the calyx, and the volume of the stamens changed in response. After 800 h of chilling, the number of stamens increased, the volume increased, and the stamen primordia differentiated, forming filaments and anthers, and the anther primordia began to expand, forming the basic structure of anthers. The development state of L12 flower buds was always lagging behind that of N1. Therefore, peach blossom buds exposed to low temperature developed faster in varieties with a low chilling requirement.

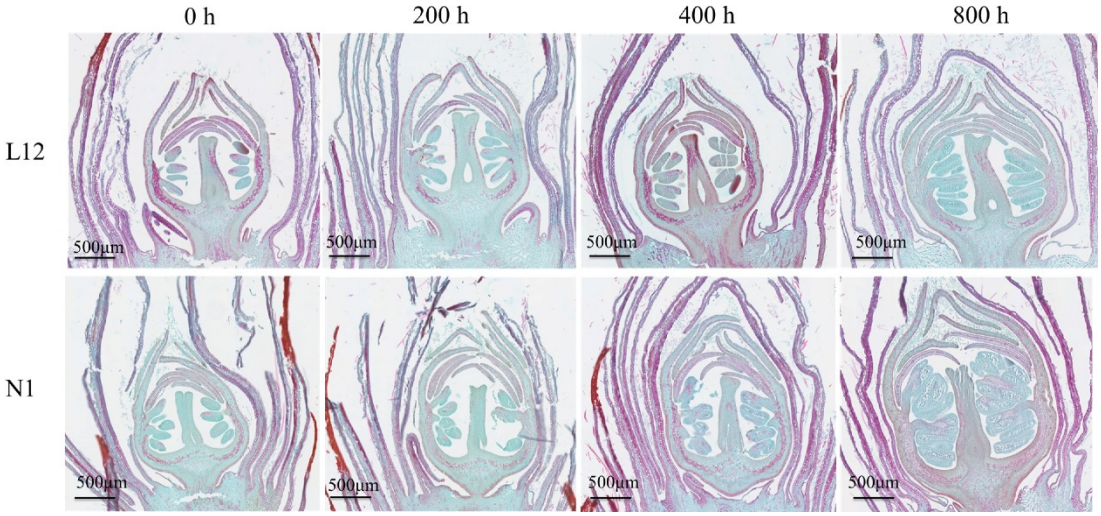

Figure 1. Internal development state of flower buds under different chilling hours.

3.2. Characteristics of Changes of Hormone Content in Flower Buds with Chilling Requirement

As illustrated in Figure 2a, the IAA content under the chilling hours of 0-800 exhibited a trend of initially increasing and then decreasing, reaching its peak at 400 h. The content of L12 was the lowest at 0 h, reached the peak at 400 h, and then began to decline. The IAA

content of N1 increased significantly at 200 h to break the dormancy state and decreased at 800 h, but the decrease was relatively small.

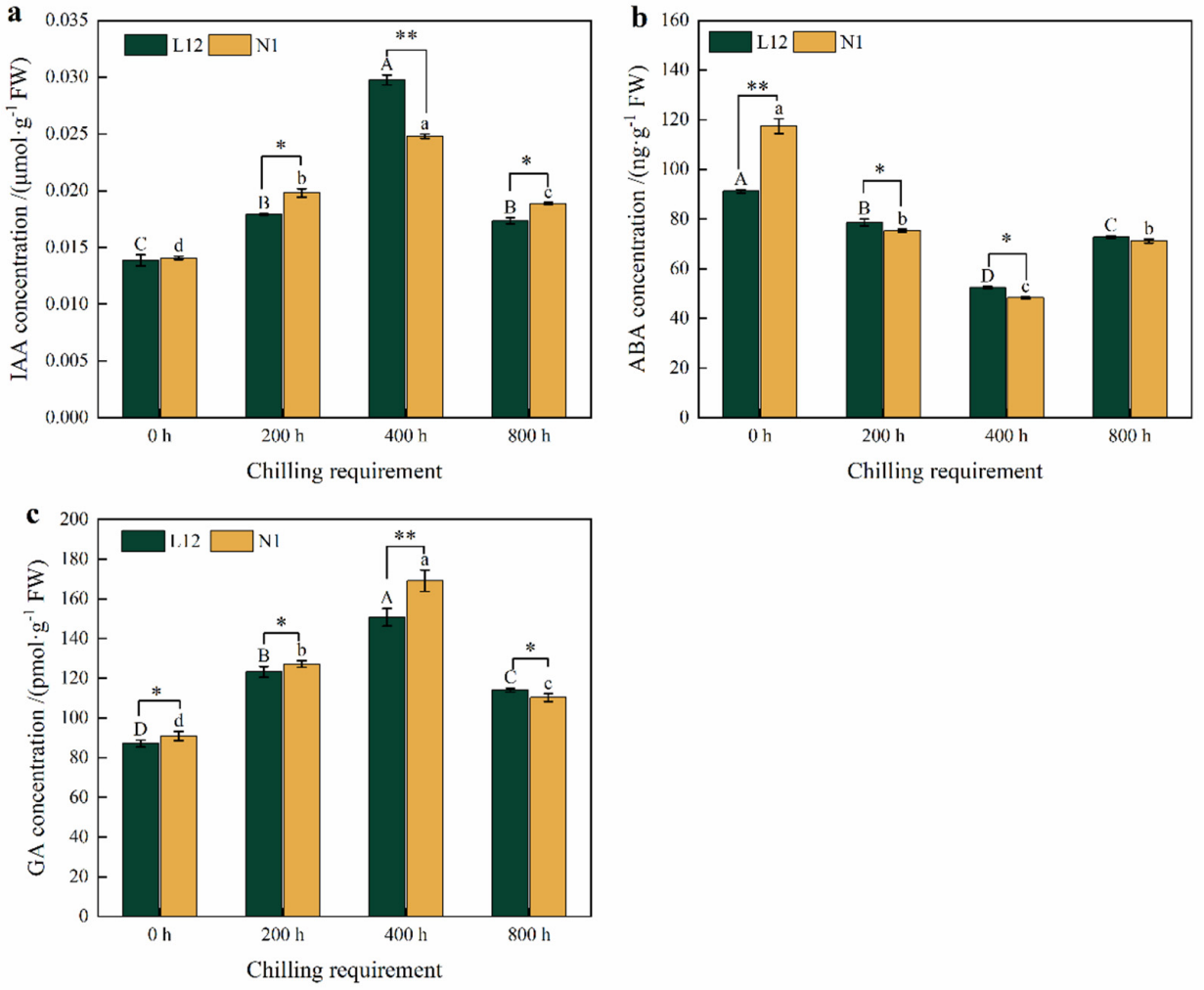

Figure 2. Contents of IAA (**a**), ABA (**b**), and GA (**c**) at different chilling hours. Different uppercase and lowercase letters indicate significant differences between treatments at a $p < 0.05$ level. Different * indicates significant differences between samples of different varieties (*, $p < 0.05$; **, $p < 0.01$, t test).

As illustrated in Figure 2b, ABA content initially decreased and then increased with the rising chilling hours from 0 to 800. At 400 h, ABA content reached its lowest point, demonstrating a complementary trend with IAA content. At 0 chilling hours, ABA content was the highest among all treatments, with a significant difference observed between N1 and L12, with N1 being 17.8% higher than L12. At 200 and 400 h, the ABA content in the N1 treatment decreased rapidly.

As shown in Figure 2c, the GA content under the chilling hours of 0–800 exhibited a trend of initially increasing and then decreasing, mirroring the pattern observed for IAA, with GA content peaking at 400 h. At 0 chilling hours, the GA content of N1 was 90.97 pmol·g^{-1}. At 400 h, the GA contents of L12 and N1 reached their highest values, with significant differences observed compared to other stages of chilling hours, measuring 150.73 and 169.12 pmol·g^{-1}, respectively. The GA content began to decrease after 800 h.

3.3. Transcriptome Sequencing Analysis of L12 and N1

To analyze the flower bud differentiation mechanisms of the "Nanguitao 1" (N1) and "Longmi 12" (L12) varieties under different chilling hours, three replicates were selected for each variety, with transcriptomic analysis conducted across four chilling hours (0 h,

200 h, 400 h, 800 h). Among the 24 samples, each contained clean data of 5.70 Gb, with clean reads ranging from 19,892,957 to 23,543,930. The average clean reads for L12 and N1 were 20,444,877 and 20,471,971, respectively. The percentage of Q30 bases was 93.71% and above (Table S1), significantly exceeding the quality assessment standards (Q20 > 90% and Q30 > 80%). The sequencing data were of high quality and suitable for downstream analysis. To ensure the accuracy of subsequent analyses, we performed strict quality control on the data by removing reads containing adapters and low-quality reads (including those with more than 10% ambiguous bases). Clean reads from each sample were sequentially compared to the designated reference genome (Prunus_persica.Chinese_Cling_v1.0.genome.fa), with comparison efficiency ranging from 90.95% to 97.31%.

3.3.1. Differential Expression Analysis of L12 and N1 Genes

Differentially expressed genes were screened based on the count values of genes in each sample using BMKCloud (www.biocloud.net). "Longmi 12" (L12, 700 h), exhibiting the highest chilling requirement, was selected as the control, while "Nanguitao 1" (N1, 150 h), with the lowest chilling requirement, served as the test group. To further analyze the specificity of differential gene expression between the two varieties under different chilling hours, principal component analysis (PCA) revealed good repeatability among the samples. L12 and N1 exhibited significant clustering under varying chilling hours, indicating higher levels of differential gene expression in the two varieties (Figure 3a). Differential analysis was conducted using DESeq2 software (R4.1.2), with fold change ≥ 1.5 and FDR < 0.01 established as screening criteria. A total of 2618 differentially expressed genes were identified in L12 and N1 under 0 chilling hours, with 1651 genes up-regulated and 967 genes down-regulated. Under 200 chilling hours, 3592 differentially expressed genes were identified between L12 and N1, with 2233 genes up−regulated and 1359 genes down-regulated. Under 400 chilling hours, 4719 differentially expressed genes were identified, with 2923 genes up-regulated and 1796 genes down-regulated. Under 800 chilling hours, a total of 2685 differentially expressed genes were identified in L12 and N1, with 1712 genes up-regulated and 973 genes down-regulated. Additionally, the number of up−regulated genes exceeded that of down-regulated genes (Table 1).

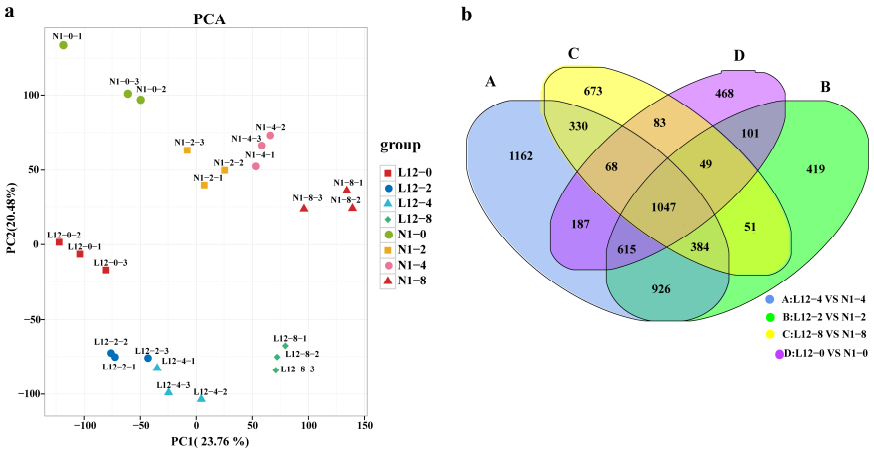

Figure 3. Principal component analysis (PCA) (**a**) and Venn diagram of differential gene set (**b**). The numbers on each region in (**b**) represent the number of genes under the corresponding classification, where overlapping regions represent the number of differential genes shared among related combinations in the region.

Table 1. Statistical table of the number of differentially expressed genes.

DEG Set	DEG Number	Up-Regulated	Down-Regulated
L12-0_vs._N1-0	2618	1651	967
L12-2_vs._N1-2	3592	2233	1359
L12-4_vs._N1-4	4719	2923	1796
L12-8_vs._N1-8	2685	1712	973

To visually illustrate the overlap of differentially expressed genes among various comparison groups, a Venn diagram was utilized. The Venn diagram allowed for the screening of common and unique differential gene sets among specific comparison groups. It was determined that L12 and N1 shared 1047 common genes across four different chilling hours, with 468 specific genes identified at 0 h, 419 specific genes at 200 h, 1162 specific genes at 400 h, and 673 specific genes at 800 h. At 400 h, the highest number of unique differentially expressed genes was identified between the two varieties, highlighting specific differential expression at this chilling stage (Figure 3b).

Genes exhibiting similar expression patterns may share functional roles. To visually represent the expression differences of genes across various groups and to identify potential functional genes, hierarchical clustering analysis was conducted on all differentially expressed genes, clustering those with similar expression patterns across samples. The heat map depicting gene clustering expression is presented in Figure 4. Samples of L12 and N1 with similar expression levels of differential genes were clustered together with closely related branches. The patterns of differential gene expression were similar, suggesting that the functions of genes within this cluster are analogous.

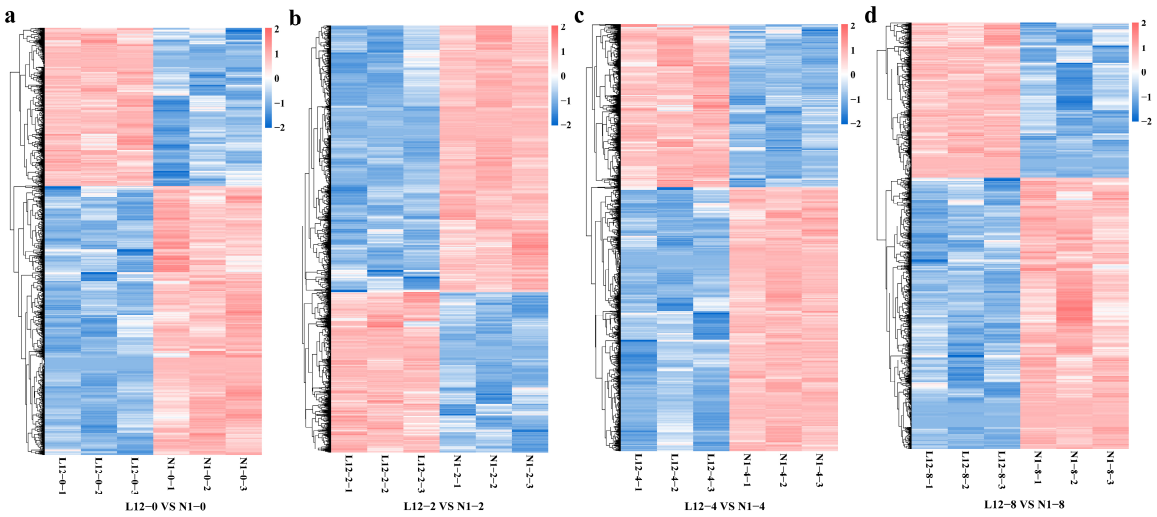

Figure 4. Clustering heatmap of differentially expressed genes in L12 and N1 at 0 (**a**), 200 (**b**), 400 (**c**), and 800 (**d**) chilling hours. The horizontal coordinate represents the name of the sample and the clustering result of the sample, and the vertical coordinate represents the differential gene and the clustering result of the gene. Different columns in the diagram represent different samples, and different rows represent different genes. The colors represent the level of gene expression in the sample log10 (FPKM + 0.000001).

3.3.2. Enrichment Analysis of L12 and N1 Differential Genes

Functional annotation was performed on the differential expression gene database. The number of genes annotated for each differential expression gene set is presented in Table 2 for 0 chilling hours. A total of 2519 differentially expressed genes were annotated, with 866 genes annotated in the COG database. Additionally, 2057 differentially expressed genes were annotated in the GO database, 1736 in the KEGG database, 1225 in the KOG database, 2515 in the NR database, and 2007 in the Pfam database. The Swiss-Prot database

annotated 1825 differentially expressed genes, while the eggNOG database annotated 2040. Compared to other time points, the number of differentially expressed genes annotated in each database at 400 h was greater than at the other time points. At 400 h, a total of 4557 differentially expressed genes were annotated in the COG database, 1565 in the GO database, and 3779 in the KEGG database. At 0 h, the number of differentially expressed genes annotated in the GO database exceeded that in the COG database by 1729, the KEGG database had 3141 more than the GO database, and the KOG database had 2222 more than its own annotation count. A total of 4555 differentially expressed genes were annotated in the NR database, 3691 in the Pfam database, 3325 in the Swiss-Prot database, and 3762 in the eggNOG database.

Table 2. Statistical table of the number of differentially expressed genes annotated.

DEG Set	Total	COG	GO	KEGG	KOG	NR	Pfam	Swiss-Prot	eggNOG
L12-0_vs._N1-0	2519	866	2057	1736	1225	2515	2007	1825	2040
L12-2_vs._N1-2	3466	1193	2856	2362	1677	3463	2795	2551	2850
L12-4_vs._N1-4	4557	1565	3779	3141	2222	4555	3691	3325	3762
L12-8_vs._N1-8	2569	911	2080	1739	1225	2566	2064	1852	2051

(1) GO classification enrichment analysis

The GO annotation system was utilized to classify the differentially expressed genes at the secondary classification level of the GO database, and each GO term in the three categories of biochemical processes, cellular components, and molecular functions were counted. At 0 chilling hours, L12 and N1 annotated a total of 2057 differentially expressed genes across three subdivisions. The cellular and metabolic processes, with 1017 and 897 differentially expressed genes, accounted for the majority of annotations in these categories. The two most represented groups of cellular components were the cellular anatomical entity and intracellular categories, with 1301 and 686 annotations, respectively (Figure 5). The two most clustered groups of molecular functions were binding and catalytic activities, with 1029 and 1001 differentially expressed genes annotated, respectively. Under 200 chilling hours, L12 and N1 annotated a total of 2856 differentially expressed genes across three subdivisions. In biochemical processes, 1465 differentially expressed genes were observed in cellular processes and 1207 in metabolic processes. The two groups that clustered most in cellular components were the anatomical entity and intracellular categories, with 1877 and 1038 annotations, respectively. In molecular functions, binding and catalytic activities were the two most clustered groups, with 1447 and 1333 differentially expressed genes annotated, respectively. Under 400 chilling hours, L12 and N1 annotated a total of 3779 differentially expressed genes across three subdivisions. In biochemical processes, 1970 differentially expressed genes were annotated in cellular processes and 1685 in metabolic processes. For cellular components, 2492 annotations were obtained for the anatomical entity and 1402 for intracellular categories. Additionally, 1873 and 1771 differentially expressed genes were annotated for binding and catalytic activities, respectively, with the highest concentration in molecular functions. Under 800 chilling hours, L12 and N1 annotated a total of 2080 differentially expressed genes across three subdivisions. A total of 1041 and 916 differentially expressed genes were annotated in cellular processes and metabolic processes, respectively, which were the most prominent in biochemical processes. The anatomical entity annotation for the most clustered group in cellular components was 1309; additionally, 1004 and 1030 differentially expressed genes were annotated for binding and catalytic activities in molecular functions. Notably, biological processes such as cellular processes, cellular anatomical entities, and binding of DEGs were significantly more abundant at 400 h of chilling than at other cooling periods, indicating substantial enrichment of DEGs at this stage. This analysis confirmed that signal transduction processes were overexpressed in DEGs at 200 and 400 chilling hours.

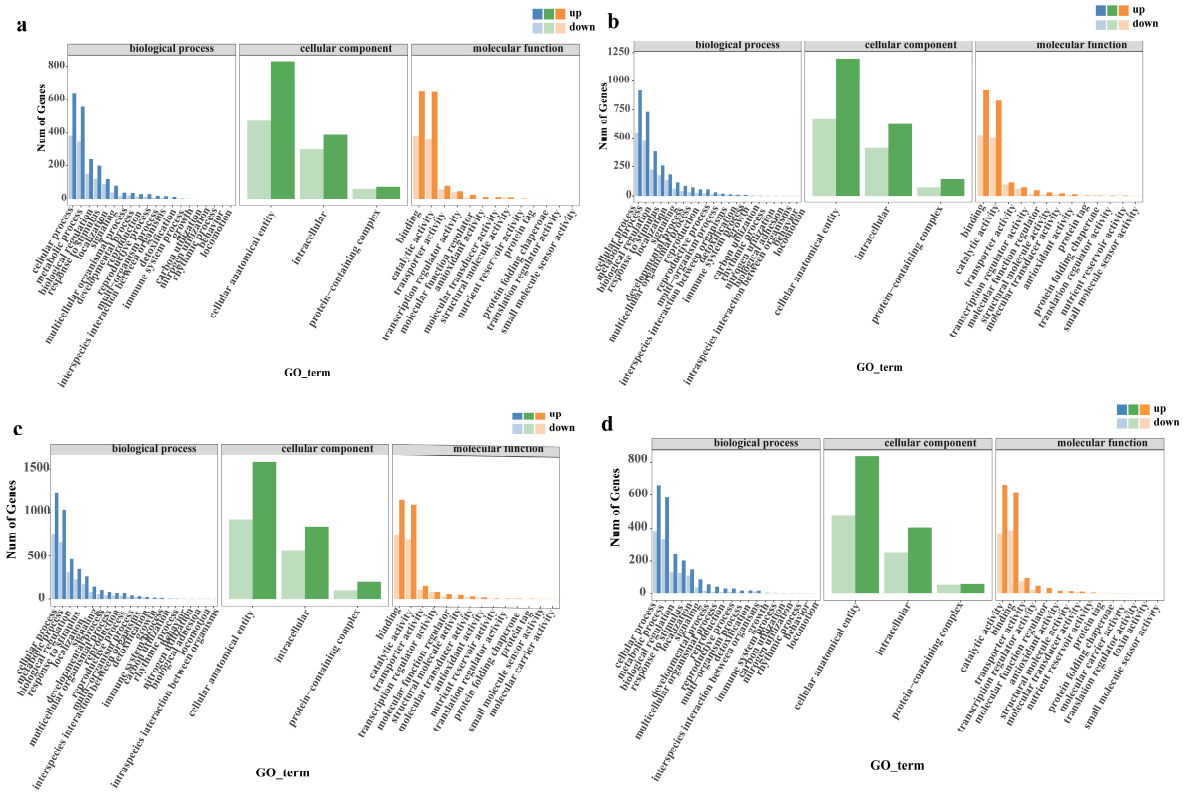

Figure 5. GO mapping of differentially expressed genes of L12 and N1 at 0 (**a**), 200 (**b**), 400 (**c**), and 800 h (**d**). The horizontal coordinate is the GO classification, the vertical coordinate is the number of genes, and different colors represent different primary classification.

(2) KEGG classification notes

Additionally, we conducted KEGG pathway analysis on differentially expressed genes and found that these genes were classified into five categories: cellular processes, environmental information processes, heredity, metabolism, and organic systems (Figure 6). During different chilling periods, the differential genes of L12 and N1 were mainly enriched in pathways such as plant hormone signal transduction, MAPK signaling pathway, plant–pathogen interaction, starch and sucrose metabolism, and protein processing in the endoplasmic reticulum. The differentially expressed genes for L12 and N1 at the four chilling hours (0 h, 200 h, 400 h, 800 h) were 1225, 1677, 2222, and 1225, respectively. Among these, the most enriched pathways for the differentially expressed genes of L12 and N1 at 0 chilling hours were plant hormone signal transduction (84 genes), plant MAPK signaling pathway (65), starch and sucrose metabolism (100), and plant–pathogen interaction (130). At 200 chilling hours, the most abundant pathways for L12 and N1 in the KEGG analysis included endocytosis (47 genes), plant hormone signaling (116), plant MAPK signaling (75), starch and sucrose metabolism (81), and plant–pathogen interaction (149). At 400 chilling hours, the most enriched pathways for L12 and N1 were plant hormone signal transduction (140 genes), plant MAPK signaling pathway (100), starch and sucrose metabolism (100), and plant–pathogen interaction (190). At 800 chilling hours, the most notable pathways for L12 and N1 included plant hormone signal transduction (67 genes), plant MAPK signaling pathways (52), phenylpropanoid biosynthesis (56), starch and sucrose metabolism (55), and plant–pathogen interactions (98).

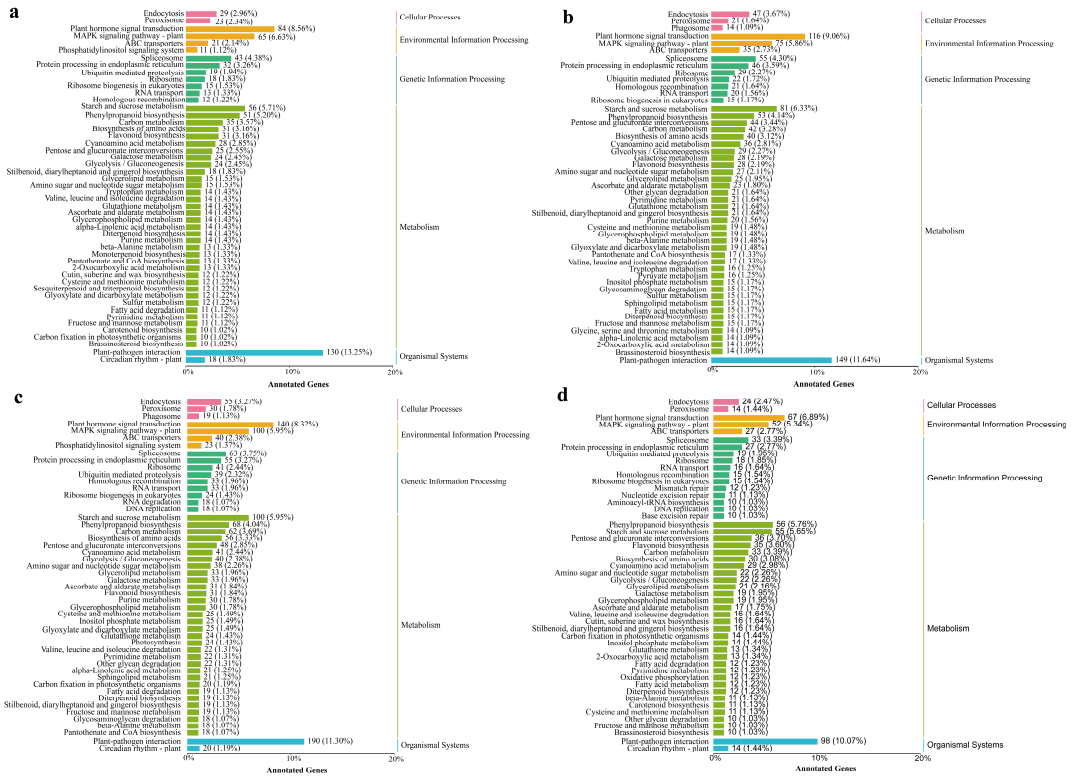

Figure 6. KEGG classification map of differentially expressed genes. The left ordinate represents the name of the KEGG metabolic pathway, the right ordinate represents the first-class classification name corresponding to the annotated pathway, and the horizontal coordinate represents the number of genes annotated to the pathway and their proportion to the total number of annotated genes. Figures (**a**–**d**) represent L12-0 VS N1-0, L12-2 VS N1-2, L12-4 VS N1-4, and L12-8 VS N1-8, respectively.

Of these, 140 differentially expressed genes in the plant hormone signal transduction pathway reached their peak at 400 chilling hours, accounting for 8.32% of all signal pathways and representing increases of 66.67%, 20.69%, and 108.96% compared to 0, 200, and 800 h, respectively. Specifically, there were 44, 19, and 44 more differentially expressed genes in starch and sucrose metabolism at 400 h compared to at 0, 200, and 800 h, respectively. This suggests that specific transcription factors or regulatory networks may be responsible for the activation or inhibition of additional genes at 400 h.

Hypergeometric tests were conducted to identify pathways with significant enrichment of differentially expressed genes compared to the entire genomic background, using a significance threshold of less than 0.05. The key biochemical, metabolic, and signal transduction pathways associated with the genes were identified through significant pathway enrichment. We identified five pathways with high q-values and their associated genes. At 0 h, the five pathways with the most enriched differentially expressed genes for L12 and N1 were flavonoid biosynthesis, galactose metabolism, the MAPK signaling pathway, phenylpropanoid biosynthesis, and plant–pathogen interaction. At 200 h, the five pathways with the highest q-values for differentially expressed genes in L12 and N1 were ABC transporters, cyano amino acid metabolism, plant hormone signal transduction, starch and sucrose metabolism, and β-alanine metabolism. Under 400 h, the five pathways with the most enriched differentially expressed genes for L12 and N1 were ABC transporters, ascorbic acid and uronate metabolism, photosynthesis, plant hormone signal transduction, and starch and sucrose metabolism. At 800 h of chilling, the five pathways with the highest concentration of differentially expressed genes for L12 and N1 were anthocyanin biosynthesis, lignin and wax biosynthesis (including cutin, suberin, and wax), cyanamide amino acid metabolism, flavonoid biosynthesis, and phenylpropanoid biosynthesis. At both 200 and 400 h of chilling, the number of genes of interest annotated in the hormone

signal transduction pathway increased significantly, with extremely significant q-values (Figure 7).

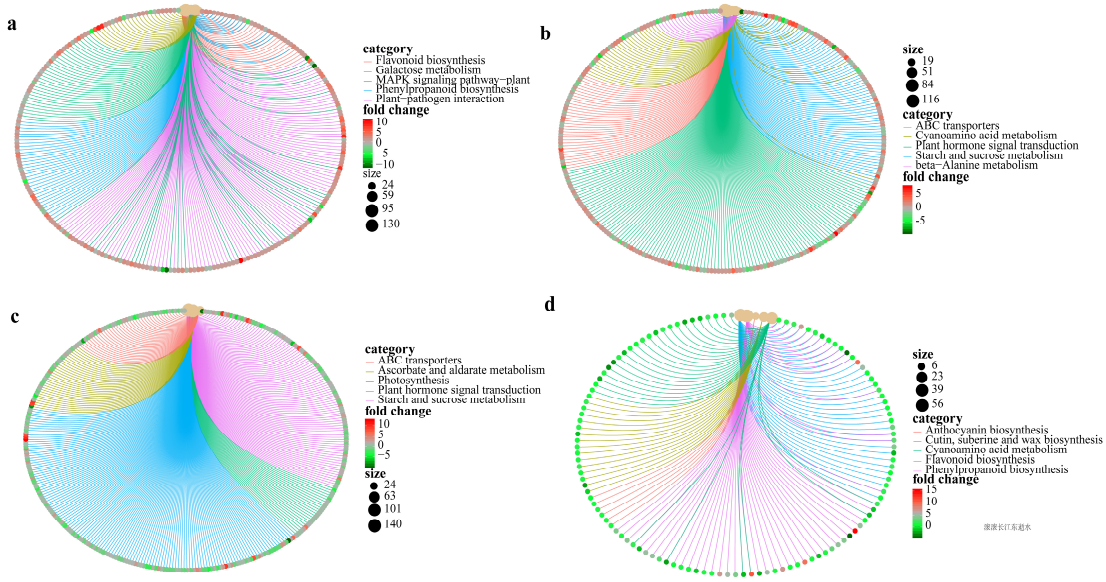

Figure 7. KEGG enrichment network of differentially expressed genes. Network diagram of differentially expressed genes and KEGG pathway. The colors of the edges represent different pathways, and the colors of gene nodes represent multiples of difference. The larger the pathway nodes are, the more genes are enriched in the pathway. Figures (**a**−**d**) represent L12-0 VS N1-0, L12-2 VS N1-2, L12-4 VS N1-4, and L12-8 VS N1-8, respectively.

3.3.3. Identification of DEG, a Hormone Pathway Involved in Flower Bud Differentiation

The molecular mechanism of flower bud differentiation involves the regulation of multiple genes, which are closely linked to hormone synthesis and signal transduction. KEGG annotation results indicated that the number of genes of interest annotated in the hormone signal transduction pathway significantly increased at 200 and 400 chilling hours. Therefore, we further analyzed the expression levels of differentially expressed genes in the hormone pathways across the four chilling hours. In the four chilling hours, we identified 72 differential genes (Figure 8) and 34 degs in the IAA pathway, among which, at the 400 h chilling requirement, IAA key gene *Prunus persica* auxin-induced gene *IAA6* (evm.TU.contig286.243), *Prunus persica* auxin-responsive protein gene *IAA27* (evm.TU.contig286.340), putative indole-3-acetic acid-amido synthetase, the expression levels of *GH3.9* (evm.TU.contig280.375), and auxin-responsive gene *SAUR50* (evm.TU.contig268.510) were significantly up-regulated in N1 treatment compared with L12 to regulate the growth and development of buds (See Table S2). In the process of GA, there were 21 degs. Among the key GA genes, DELLA protein synthesis genes *DWARF8* (evm.TU.contig163.500), *SHORT-ROOT* (evm.TU.contig284.178), and transcription factor *BIM1* (evm.TU.contig278.1353) were significantly upregulated in N1 at 400 chilling hours. A total of 17 degs were identified in the ABA pathway. Among them, 14 degs were down-regulated at 400 chilling hours. Interestingly, *PYL2* (evm.TU.contig272.954) and *PYL8* (evm.TU.contig270.213), which can act as ABA receptors to regulate gene expression and promote flower germination, were always at a low level in N1 treatment. The expression level of L12 was the lowest at 400 h, and the expression level of L12 was 3.58 times and 1.67 times that of N1. Other ABA pathway-related genes include 2C family protein phosphatase *2C 24* and *2C 54*, *ABSCISIC ACID-INSENSITIVE 5-like* (evm.TU.contig272.765), serine/threonine protein kinase *SRK2A* (evm.TU.contig132.21), and mume serine/threonine protein kinase *SAPK2-like* (evm.TU.contig272.20), which were expressed at a low level at 400 chilling hours of N1.

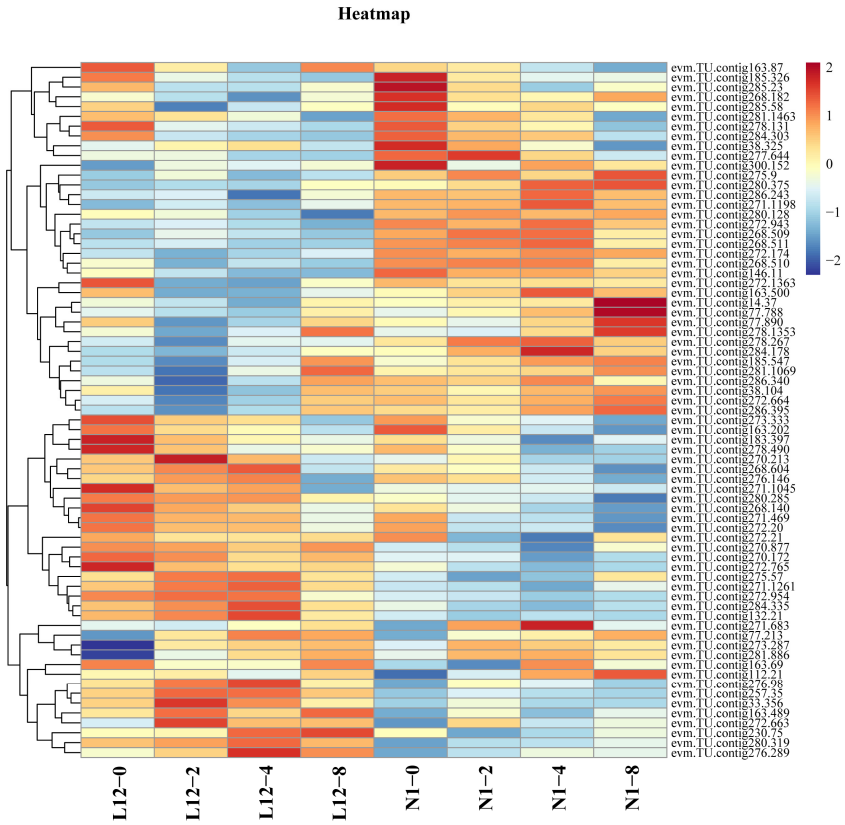

Figure 8. Analysis of differential genes of L12 and N1 hormone pathways.

3.4. Transcriptome Analysis of L12-8 and N1-2

The chilling requirement of L12 amounts to 700 h, while that of N1 is 150 h. Theoretically, when both L12 and N1 fulfill their respective chilling requirements, the morphology, physiology, metabolic pathways and genes of flower buds will change. Therefore, we compared L12, which met the chilling hour requirement at 800 h, with N1, which met the chilling hour requirement at 200 h. It was discovered that there were 2717 differential genes between L12-8 and N1-2, among which 1827 genes were up-regulated in N1-2 and 890 genes were down-regulated. Notably, the number of up-regulated genes was significantly higher than that of down-regulated genes.

3.4.1. KEGG Classification Annotations of L12-8 and N1-2

We performed functional annotation of the differential genes of L12-8 and N1-2 in the database. A total of 1678 differential gene annotations were found in the KEGG database, and further KEGG classification annotation was conducted. The results indicated that 588 differential genes were screened out in all pathways, and these differential genes were classified into five major blocks: cellular processes, environmental information processes, genetic information processing, metabolism, and organismal systems (Figure 9). Moreover, at different chilling hours, the differential genes of L12-8 and N1-2 were mainly enriched in plant–pathogen interaction, plant hormone signal transduction, MAPK signaling pathway for plants, and starch and sucrose metabolism pathway. Respectively, 96, 44, 35, and 37 differentially expressed genes were annotated. Plant–pathogen interaction and plant hormone signal transduction accounted for 16.33% and 7.48% of all paths, respectively.

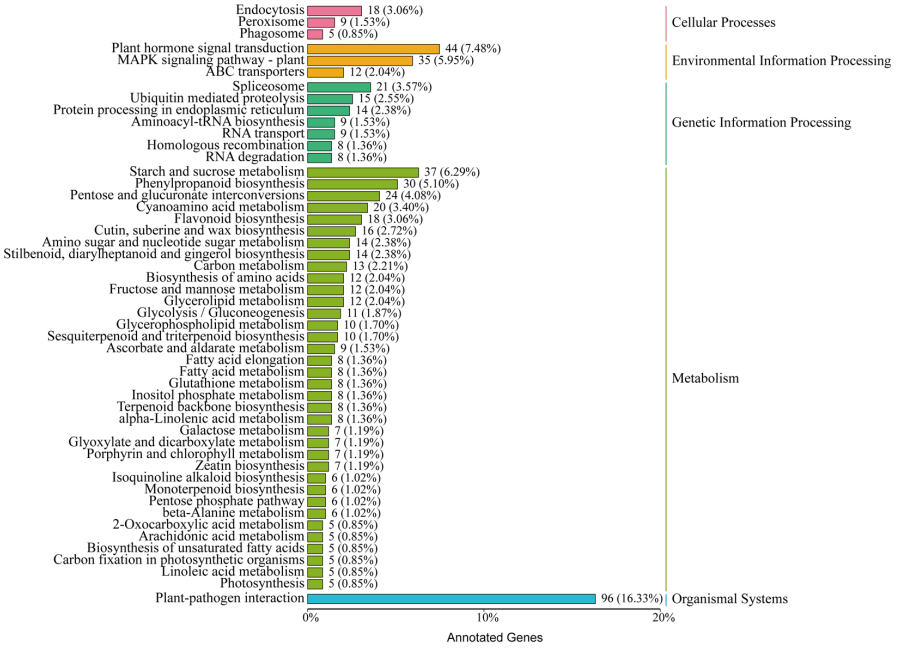

Figure 9. KEGG classification annotation for L12-8 and N1-2.

3.4.2. Identification of DEGs in the L12-8 and N1-2 Hormone Pathways

KEGG annotation showed that L12-8 and N1-2 had the highest number of genes in the plant–pathogen interaction pathway and the highest number of genes of interest in the plant hormone signal transduction pathway. Therefore, we further analyzed the differential gene expression levels of L12-8 and N1-2 in the hormone pathway. We identified 16 DEGs involved in IAA, GA, and ABA pathways (Figure 10), including IAA key gene Prunus persica auxin-induced protein gene IAA6 (evm.TU.contig286.243), auxin-responsive gene SAUR50 (evm.TU.contig268.509, evm.TU.contig268.510, evm.TU.contig268.511, evm.TU.contig271.1198, and evm.TU.contig284.303), auxin response factor 18 (evm.TU.contig277.644), auxin-responsive SAUR36 (evm.TU.contig278.131), indole-3-acetic acid-amido synthetase gene GH3.1 (evm.TU.contig185.326), GA key gene CXE15 (evm.TU.contig146.11), scarecrow-like 28 (evm.TU.contig278.267), PRUPE (evm.TU.contig276.146), and ABA key gene 2C 72 (evm.TU.contig272.943).

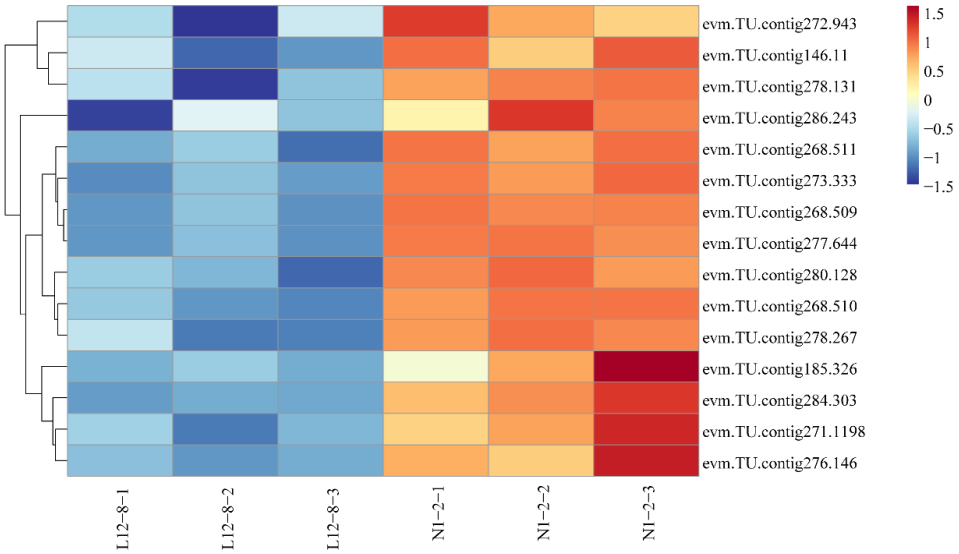

Figure 10. Analysis of differential genes of L12-8 and N1-2 hormone pathways.

3.5. Transcription Factor Analysis of L12 and N1

Understanding the role of transcription factors in the regulatory network of flower bud development is critical; the GO term "nucleic acid-binding transcription factor activity" indicates that these factors significantly influence flower bud differentiation. Therefore, we extracted differentially expressed genes (DEGs) from the flower buds of L12 and N1 across four chilling hours to identify transcription factors involved in peach blossom bud development. We identified 1291 genes encoding transcription factors, encompassing 20 distinct families. Among the DEGs, the most prominent transcription factor families were *AP2_ERF-ERF*, *bHLH*, and *C2H2*, accounting for 8.37%, 8.29%, and 7.67% of the differentially expressed transcripts, respectively (Figure 11). Other family transcription factors, such as *MYB* and *NAC* transcription factors, are also common. Notably, *AP2_ERF-ERF* and *MADS* transcription factors are critical for flower organ formation, regulating the development of petals, stamens, and pistils, and may play a role in the flower bud development of this species, making them promising candidate genes.

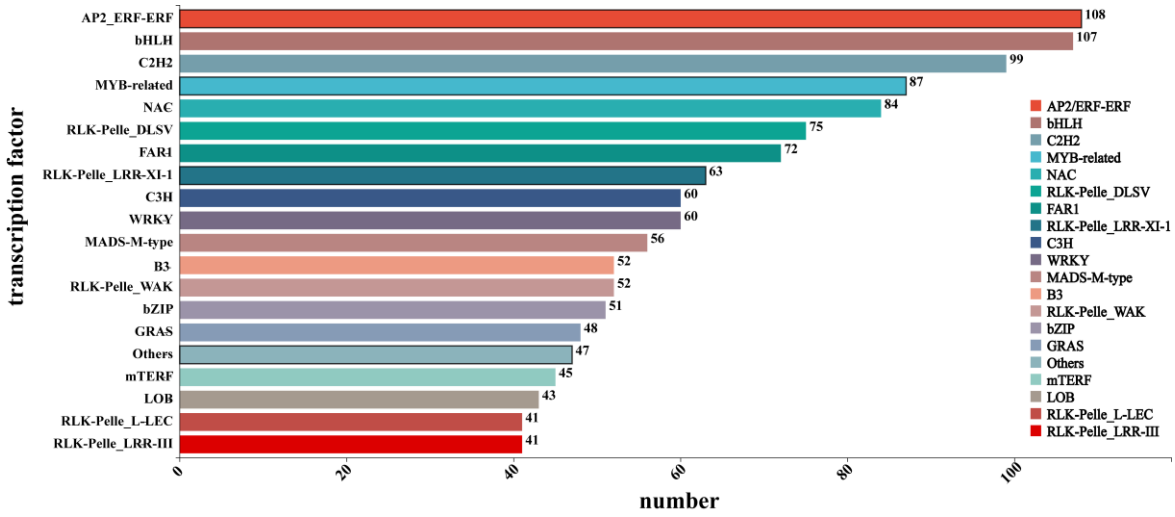

Figure 11. Transcription factor analysis of differentially expressed genes.

4. Discussion

4.1. The Differentiation of Flower Buds Was Influenced by Hormonal Levels, with Significant Impacts from the Concentrations of IAA, ABA, and GA Observed Under Different Chilling Hours

Flower bud differentiation is a key physiological process that determines the yield and quality of peach trees [7]. This process is significantly influenced by environmental factors (temperature, light, moisture), plant hormone regulation, genetic mechanisms, and other factors [22]. Flower bud differentiation requires the accumulation of low temperatures below 7.2 °C, known as the chilling requirement [23]. Studies have shown that insufficient chilling requirements can result in incomplete flower bud differentiation, affecting both flowering time and fruit yield [24]. This study utilized the low chilling requirement variety N1 as the test material, designing four chilling hours of 0, 200, 400, and 800, while using L12, with a relative chilling requirement of 700 h, as the control. In the 4 chilling hours of flower bud differentiation, the paraffin section map of flower bud longitudinal cutting was observed. It was found that N1 flower buds developed rapidly under 400 chilling hours, their volume became significantly larger, and the volume and number of stamens became larger. The volume and number of stamens of L12 increased when the chilling hour requirement was 800. The results showed that N1 needed more chilling hours to change morphologically after satisfying its own chilling requirements.

Hormones are the primary factors regulating flower bud differentiation, and significant differences exist in the types and levels of hormones at various developmental stages of flower buds [25–27]. As chilling hours increased, peach flower buds gradually entered the differentiation stage, during which the levels of plant hormones, such as IAA and GA, began

to rise, promoting flower bud development [28]. It was observed that, at this stage, the differentiation rate of varieties with low chilling requirements was faster, while those with high chilling requirements remained in the low-temperature accumulation phase [29]. At this stage, the influence of hormone balance on flower bud differentiation became apparent, particularly the interaction between auxin and gibberellin, which peaked, while abscisic acid levels remained relatively low [30]. This indicates that hormone balance is crucial for flower bud differentiation [31]. Three main plant hormones were identified in the flower buds of the two varieties across the four chilling hours in this study. IAA levels initially increased and subsequently decreased during the four chilling hours, reaching the highest concentration at 400 chilling hours. IAA promotes the elongation and growth of in vitro coleoptiles and young stem segments, facilitates vascular bundle differentiation, enhances competitiveness, and influences the activity of IAA catabolism-related enzymes, ultimately affecting flower bud differentiation [32,33]. The results indicate that low-temperature treatment enhances flower bud differentiation, increases the activity of IAA catabolism-related enzymes, and elevates both the quantity and quality of pollen by increasing the frequency of pollen mother cell division [34]. Additionally, it promotes the growth and development of both the pistil and the stamen, leading to more well-formed structures [35]. The results of this study were similar to those of previous studies, although the IAA content of N1 treatment was less than L12 at 400 chilling hours. GAs enhance the rate of plant cell division and expansion, which are crucial for the transition to flowering and the formation of flower primordia [36]. In this study, the change trend of GA content was the same as that of IAA, and it reached the highest level at 400 chilling hours. Moreover, according to the state of paraffin, N1 broke the dormancy of buds and promoted the morphological differentiation of flower organs. Furthermore, the ABA content of N1 and L12 decreased to the lowest level until 400 h. This suggests that the inhibitory effect of N1 on flower bud differentiation was diminished [37]. Nevertheless, the regulation of hormonal balance is complex and variable, highlighting the critical role of gene regulation.

4.2. Transcriptome Analysis Revealed That the Differentiation of Flower Buds Under Varying Chilling Hours Is Governed by the Gene Balance Across Multiple Hormonal Pathways

This study utilized KEGG analysis to confirm the significant role of plant hormone signal transduction in both L12 and N1. Additionally, heat map analysis identified 72 differentially expressed genes (DEGs) involved in the auxin, gibberellin, and abscisic acid signaling pathways. In the auxin signaling pathway, the expression levels of *AUX1*, *LAX*, *IAA*, and *SAUR* genes were relatively high in the N1 variety under the initial chilling hours. Conversely, the expression level of the *GH3.9* gene in L12 was low, exhibiting a trend of initially increasing and then decreasing. In contrast, the expression level in N1 continuously increased and the expression level reached the highest point at 800 h of chilling. The initial expression level of *SAUR50* (evm.TU.contig268.509, evm.TU.contig268.510, evm.TU.contig284.303) in N1 was significantly higher than that in L12, and the analysis of L12-8 and N1-2 at the same physiological stage showed that the expression of *SAUR50* was significant in N1-2. The results indicated that *SAUR50* played a significant role in regulating IAA levels. *GH3.9* belongs to Group II of the *GH3* family [38], and in vitro studies have demonstrated that most members of Group II function as IAA–amide synthetases, binding amino acids to the plant hormone auxin [39]. The *SAUR* gene family primarily regulates auxin synthesis and transport, impacting cell expansion. Notably, these genes lack introns, cluster on chromosomes, and contain auxin response elements as well as downstream elements that promote mRNA degradation [40]. During the active–dormancy–active state transition of overwintering tea buds, the early auxin response gene *CsGH3.9* and the *SAUR* gene were specifically expressed in flowers. Both genes were significantly down-regulated during the deep dormancy stage of early tea varieties but exhibited strong expression stimulation prior to germination [41]. This finding aligns with the results of the current study. The changes in IAA content across the four chilling hours reflected the growth

status of peach buds during the transition from active dormancy to active differentiation of flower buds.

The regulation of flower formation by the gibberellin pathway is one of the earliest pathways identified for flower development. Gibberellins (GAs) activate the expression of genes involved in flower formation, playing a crucial role in the development of flowers in perennial woody plants [42]. *Carboxylesterase 15* (*CXE15*) belongs to the GID1 gene family and is mainly involved in the hydrolysis of ester compounds, which is usually regulated by stress responses such as drought, salt stress, and oxidative stress. The analysis of L12-8 and N1-2 showed that *CXE15* was significantly expressed in N1-2, which may be the basis for GA regulation of N1-2 development [43]. *PIF4*, a bHLH transcription factor, positively regulates cell elongation and stimulates the expression of the *DELLA* gene [44], a core negative regulator in the gibberellin (GA) signaling pathway. The ubiquitin–proteasome system alleviates growth inhibition in plants, promoting hypocotyl and stem elongation [45]. Transcriptome analysis of both vegetative and reproductive buds, as well as mature leaves of star fruit, indicated that the *PIF4* gene is involved in flower induction and regulation [46]. Results indicated that the *DELLA* gene is highly expressed in flower buds, particularly in both female and male flower buds [47]. In this study, significant differences were observed in the *BIM1* and *PIF4* genes between the N1 variety and L12. The three DELLA genes *SCARECROW*, *DWARF8*, and *SHORT-ROOT* exhibited relatively high levels during the whole chilling stage. Genes associated with the gibberellin response were identified. Furthermore, the *DELLA* gene positively influences flower bud differentiation, and the rapid development of N1 stamens was confirmed in flower bud sections.

Stress rapidly induces ABA accumulation in plants. Upon sensing ABA molecules, the ABA receptor *PYR/PYL/RCAR* interacts with its co-receptor *PP2C*, inhibiting *PP2C* phosphatase activity. This releases the inhibition of SnRK2 kinase, a key positive regulatory factor in the ABA signaling pathway [48]. SnRK2 phosphorylates and activates a series of response factors in the ABA signaling pathway, aiding plants in stress resistance [49]. During flower bud differentiation, ABA interacts with *ABF-like* transcription factors via the *SnRK2* family to regulate the expression of key genes involved in this process [50]. This study found that at the initial stage of flower bud differentiation (0 chilling hours), expression levels of most ABA signaling pathway genes, including *PYL*, *PP2C*, *SnRK2*, *ABF*, and other signaling molecules, were low in N1 and high in L12. This indicates that N1, a variety with a short chilling requirement, reduces ABA content at the initial stage, adjusting the chilling units for flower bud differentiation by lowering the gene expression levels in this pathway. The expression patterns of the *SNRK2* (evm.TU.contig132.21) and *ABF* (evm.TU.contig271.1261) genes were similar when comparing ABA signaling molecules in the transcriptomes, suggesting an interplay between *SNRK2* and *ABF* transcription factors. In conclusion, the regulation of flower bud differentiation is governed by a balance of genes across various hormone pathways, which cannot be achieved by a single hormone. Therefore, the coordination of multiple plant hormone signals is essential for achieving flower bud differentiation under varying chilling hours.

4.3. The Transcription Factor Gene Families AP2_ERF-ERF, bHLH, C2H2, MYB, NAC, and RLK Play a Crucial Role in Regulating Low-Temperature Tolerance and Flower Bud Differentiation

Additionally, GO and transcription factor analysis revealed that the most representative transcription factor families include *AP2/ERF*, *bHLH*, *C2H2*, *MYB*, *NAC*, and *RLK*. In response to low temperatures, *AP2/ERF* transcription factors regulate the expression of genes involved in flower bud differentiation by binding to specific DNA sequences, thereby promoting or inhibiting flower bud formation and development [31]. Wang [51] et al. studied sweet cherries and found that *AP2/ERF* transcription factors influence the dormancy and activation processes of flower buds in response to low temperatures. Furthermore, transcription factors such as *bHLH*, *C2H2*, and *MYB* can regulate chilling requirements and flower bud differentiation under low-temperature conditions [52]. However, our study

lacks clear evidence, suggesting a theoretical basis for further confirmation of the regulatory mechanisms underlying flower bud differentiation across varying chilling requirements.

5. Conclusions

In this study, the effects of low-temperature treatment on flower bud development were disclosed by comparing the paraffin sections and hormone alterations of L12 and N1 varieties under different chilling hours. The findings indicated that the flower bud development status of L12 was fuller than that of N1 at the same physiological stage. Under the same treatment duration, the variations in hormone levels in flower buds at 400 chilling hours were the most remarkable, suggesting that plants were most sensitive to low temperature during this period. Further transcriptome analysis demonstrated that the number of differentially expressed genes at 400 h was the highest under the same number of treatment hours. At the same physiological stage, there were 2717 distinct genes between L12-8 and N1-2. Both of them are highly enriched in plant hormone signaling pathways, suggesting that hormone signaling pathways play a crucial role in flower bud differentiation. Through in-depth analysis of differential genes within hormone pathways, we screened out several key genes, such as *SAUR*, *AUX/IAA*, *GH3*, *DELLA*, *PP2C*, *SNRK2*, and *ABF*. *SAUR50*, *IAA6*, and *scarecrow*-like *28* exist throughout the chilling hours and are differentially expressed when N1 and L12 are at the same physiological stage, which might play a significant role in regulating flower bud differentiation and its adaptability to low chilling requirement varieties. These findings not only offer a new perspective for the further study of the physiological mechanism of peach under the condition of low chilling requirement but also provide a potential molecular target for crop cultivation management and variety improvement.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10121292/s1. Table S1. Statistical table of sequencing data. Table S2. log2FC and FDR values corresponding to differentially expressed genes.

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