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Dynamic transcriptome analysis reveals an arginine decarboxylase gene in maize can enhance plant low temperature tolerance

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Abstract

Maize (*Zea mays*), originating from tropical regions, is inherently sensitive to chilling stress. A comprehensive understanding of the transcriptional regulatory networks underlying maize chilling stress response is fundamental to breeding resilient varieties. The present study conducted a time-series RNA-sequencing on maize inbred line B73 at the three-leaf stage exposed to 8°C across 16 time points. The dynamics transcriptional changes were investigated across three distinct stages: early (1, 2, 3, 4 and 8 h), adaptation (12, 16, 20, and 24 h), and long-term of low temperature (72, 76, 80, 84, 88, 92, and 96 h). A total of 22446 differentially expressed genes were detected, showing that short-term chilling caused minimal transcriptional changes in maize, whereas continuous exposure exerted a profound impact on the transcriptome. Additionally, 23,014 expressed genes were further classified into 16 distinct clusters based on their temporal expression profiles. Among these, cluster 4 best presented the early stage, cluster7 characterized the adaptation stage, and Clusters 8 and 9 (4,631 genes collectively) dominated long-term stage. Cluster 16 contained genes highly expressed during both adaptation and long-term stages. These five chilling-responsive clusters exhibited elevated proportions of transcription factors, with the AP2/EREBP, MYB, WRKY, bHLH, NAC, and GRAS families being most prominently enriched. Finally, two arginine decarboxylase genes identified from clusters 4 and 16 were proved to enhance rice chilling tolerance by promoting putrescine accumulation. Collectively, this high-resolution temporal transcriptomic dataset provides a comprehensive framework for understanding the phased regulatory networks governing chilling stress responses.

1. Introduction

Maize is a vital crop for both food and energy production, and its yield is severely affected by biotic and abiotic stress [1]. Maize originated from tropic region and therefore is inherently sensitive to cold stress [2]. Biologically, cold stress could be divided into two categories, one is chilling stress, and the other is freezing stress [3, 4]. Low temperature stress above 0°C is known as chilling stress, which significantly affects the germination and early growth of maize, particularly during early spring in temperate regions. [5]. Chilling stress occurred at the seed germination stage results to the prolonged germination time, reduced germination rate, and weaker seedling vigor, ultimately leading to a decrease in maize production[6]. At the seedling stage, chilling stress reduces maize root cell proliferation and absorption efficiency, impairs stem and leaf development, and suppresses photosynthesis as well as water and nutrient uptake. These temperature-sensitive processes directly affect maize growth, leading to symptoms such as stem tip browning, seedling yellowing, necrosis, leaf curling, stunting, or even growth arrest and death [2]. Thus, deciphering transcriptional regulatory mechanisms and key genes that respond to chilling temperature in maize and other plants is of great significance.

Exerting significant adverse effects on plants, chilling stress damages plasma membrane structure, impairs photosynthetic capacity, and triggers excessive accumulation of reactive oxygen species (ROS), thereby resulting in retarded or even arrested plant growth and development [7, 8]. To encounter and adapt chilling stress, plants evolved diverse physiological and morphological changes, ultimately

resulting in significant alterations in gene expression, membrane lipid composition, cellular water uptake, membrane fluidity, and the accumulation of small molecules [9–11]. Transcription factors plays important roles in chilling stress response in plants, binding to the cis-element in the promoter of cold-responsive genes, thus forming complex and fine regulatory systems. C-repeat Binding Factor (CBF), also known as dehydration responsive element (DRE) binding factor1 (DREB1), have been recognized as crucial transcription factors playing irreplaceable roles in cold stress response [12, 13]. CBFs were key transcription factors regulating cold-responsive gene expression [12]. Besides the CBF genes, many other transcription factors, such as Myeloblastosis(MYB), Basic leucine zipper (bZIP), heat shock transcription factor (HSFs) and WRKY [10, 13] also contribute to the acquisition of cold tolerance by modulates the expression of cold-responsive genes. For example, the *HOS9* gene, which encoding a homeodomain transcription factor, is important for a part of cold-stress tolerance in Arabidopsis [14]. Through the genome-wide association study (GWAS), a B-class heat shock transcription factor ZmHsf21 was identified to enhance cold tolerance at both the seedling and germination stages in maize, by regulating lipid metabolism homeostasis [15].

In addition to transcription factors, signaling molecules and genes encoding key enzymes for signaling molecules production, also played important roles in plant chilling stress response. A newly identified cold sensor complex, CHILLING-TOLERANCE DIVERGENCE 6 (COLD6) and osmotin-like 1 (OSM1), triggers the production of the second messenger 2', 3'-cyclic adenosine monophosphate (2', 3'-cAMP) to positively regulate chilling tolerance in rice [16]. Under chilling stress, ABA biosynthetic rate-limiting enzyme *OsNCED3* was upregulated by phosphorylated OsbHLH150, leading to increased ABA accumulation and significantly improved chilling tolerance [17]. Previous reports showed that putrescines accumulated in plants upon low temperature stress and was associated with the increased tolerance to low temperature [18–20]. One important pathway for putrescine synthesis is the decarboxylation of arginine catalyzed by arginine decarboxylase (ADC) [21]. In *Arabidopsis thaliana*, *ADC*1 and *ADC2* mutant were defects in putrescine biosynthesis and were sensitive to cold stress [19]. In potato, the expression level of *ADC1* was found to be in accordance with the accumulative pattern of pytrescine and exhibit important roles in cold-acclimated freezing tolerance [22].

In maize, though several chilling tolerance genes were identified[5, 15, 23], our knowledge of the transcriptional regulatory networks governing maize chilling response remains largely elusive. Elucidation such regulatory networks is essential for discovering key chilling tolerance genes and breeding resilient varieties. At the present study, RNA-sequencing was conducted on a time-series of maize subjected to chilling stress. Maize inbred B73 plants at the three-leaf stage were exposed to 8℃ across 16 time points. To minimize photoperiod effects and ensure experimental reliability, samples were collected every four hours across two diurnal cycles prior to chilling treatment (designated N0–N20) and were used as control. The global transcriptome changes were firstly systematically investigated and differentially expressed genes were identified across three distinct stages: early low temperature (1, 2, 3, 4 and 8 h), low temperature adaptation (12, 16, 20, and 24 h), and long-term low temperature (72, 76, 80, 84, 88, 92, and 96 h). In addition, all expressed genes were classified to different gene clusters according gene expression profiles to better deciphering genes in chilling stress

responsive groups. Finally, two genes identified from chilling stress responsive gene clusters were functionally verification by heterologous expressed in rice.

2. METHODS

2.1 Plant Material Collection and RNA Sequencing

The maize (*Zea mays*) inbred line B73 seeds were planted in mixed soil (black soil: vermiculite = 2:1) and placed in a maize incubator for cultivation. The cultivation environment in the incubator was 25℃, 16 hours of light and 8 hours of darkness. Water frequently and observe the growth conditions. The leaves were collected and frozen immediately in liquid nitrogen and stored at -80℃ before processing. Three biological replicates were set up for each of the time points. The RNA extraction and transcriptome bioinformatics analysis of maize seedlings in this study were all done in the Nextomics Biosciences company.

2.2 Trimming and mapping of reads

Illumine sequence data were selectively filtered to remove low-quality reads. Clean reads were mapped to the B73 reference genome (Zea_mays. B73_RefGen_v4.45) using HISAT (Hierarchical Indexing for Spliced Alignment of Transcripts, http://www.ccb.jhu.edu/software/hisat) with default settings for parameters[24]. Data quality analysis was conducted by visualizing results of principal component analysis (PCA).

2.3 Time series analysis of all samples

When the sample changes over time, some genes will have similar expression trends. According to the gene expression data, they can be clustered into time-related gene clusters. Genes with the same expression pattern will be clustered into the same cluster. This article uses this method to analyze the tissue specificity between samples. The analysis software Mfuzz is a software package based on R, which can cluster genes according to similar expression profiles, which helps to find genes with similar functions[25].

2.4 Identification and functional annotation of DEGs

This experiment studies the differentially expressed genes of maize seedlings under low temperature stress. The log2Ratio (B/A) value of DEGseq is not directly calculated by the mean value of expression (FPKM) and then calculated by log, but the mRNA expression is used the local standard deviation of the quantity (counts) (according to the linear regression of all gene expression levels, take a certain cell of the corresponding gene, and then calculate the corresponding standard deviation) is standardized and then compared and calculated. When the mRNA expression level is corrected, it is related to the expression level distribution of all mRNAs in each sample. Detection method of DEGseq is based on Poisson distribution. This research project detects DEG according to Wang et al[26]. At the same time, two correction strategies are used to transform P-values into Q-values. After screening and obtaining

related genes with large functional differences and significant expression, perform a separate cluster analysis on them. KEGG pathway enrichment analysis were perform by R package clusterProfiler[27].

2.5 Plant Materials and Growth Conditions

Rice (*Oryza sativa* L. *Indica* cv. 9311) was used as the recipient for the transgenic rice. Select plump and uniformly sized seeds of rice variety 9311, ZmADC1, and ZmADC2 transgenic rice. Germinate the seeds at 35°C for 24 hours. Subsequently, transfer them to a constant temperature incubator set to 28°C during the day and 20°C at night, under a light/dark cycle of 16 h/8 h, and a humidity of 50%. After the seedlings have grown for 5–6 days, transfer them to a nutrient solution for continued growth. Once the rice seedlings reach the three-leaf stage, subject them to 8°C treatment.

2.6 Bioinformatics analysis

Using the neighbor-joining approach, a phylogenetic tree was built using the MEGA11 program (11.0.10) [28]. The Poisson model was used, and the bootstrap test consisted of 1000 iterations. The phylogenetic tree was made more aesthetically pleasing by using the chiplot online beautification tool (https://www.chiplot.online/tvbot.html).

2.7 Subcellular Localization

We created two vectors, d35s-ZmADC1-GFP (full-length CDS of *ZmADC1* with the stop codon deleted) and d35s-ZmADC2-GFP (full-length CDS of *ZmADC2* with the stop codon removed), to study the subcellular localization of ZmADC1 and ZmADC2. To create fusion proteins, the segments of these genes were cloned into the pCAMBIA1300-d35S-GFP BamH I site. Using polyethylene glycol-mediated transformation, the recombinant plasmids that were previously generated were transfected into the protoplasts of B73 etiolated seedlings, as previously mentioned. Confocal microscopy (LSM880, Zeiss) was used to detect the position of the GFP signal after the cells were cultured for 36 hours at 28°C.

2.8 RNA Extraction and RT-qPCR

The Evo M-MLV Reverse transcription premixed kit (Accurate Biotech) was used for all reverse transcription studies, and total RNA was extracted using the Trizon method. 9311, $9311^{ZmADC1\#OE1-5}$, and $9311^{ZmADC2\#OE1-10}$ lines were collected for RNA extraction to conduct gene expression study. Tissue samples were taken from the B73 inbred line's root, stem, leaf, bract, R1 leaf, primary root, aerial root, secondary root, R2-7d kernel, pollen, filament and glume shell to determine the tissue expression pattern. Using a Light Cycler R480 II, all RT-qPCR analyses were carried out with ChamQ Universal SYBR qPCR Master Mix (Vazyme, cat# Q711-02). *ZmActin* served as an internal reference for normalizing the relative gene expression.

2.9 Determination of physiological and biochemical indicators

The CAT activity and MDA content were determined using a Catalase (CAT) Activity Assay Kit (BC0200, Solarbio, Beijing, China), Malondialdehyde (MDA) Content Assay Kit (BC0020, Solarbio, Beijing, China).

The plant putrescine content was detected using a Plant Putrescine (Put) ELISA Kit (BS-E18135O2, JSBOSSEN, Nanjing, China). A UV spectrophotometer was used to determine POD and proline, and a microplate reader was used to determine SOD. Finally, use a microplate reader to measure the OD values at the corresponding wavelengths for CAT, MDA, and Put.

3. RESULTS

3.1 Transcriptome profile of 68 RNA libraries from Maize induced by low temperature at different time points

To investigate the dynamic changes in gene expression and metabolism in maize seedlings under chilling stress, a time-series RNA-seq analysis was performed using inbred line B73 at the three-leaf stage. The B73 lines at three-leaf stage were directly exposed to chilling treatment at 8°C. To ensure the reliability of the experimental results and exclude the potential influence of the photoperiod, we collected the first sample when the light period had just entered the dark period the day before low temperature treatment, which was denoted as N0. And then we gathered samples every four hours, and respectively named as N4, N8, N12, N16 and N20. Similarly, when the light period goes into the dark period for the second time, we began to treat the seedlings with low temperature (8℃) and respectively named as C0 (N24), C4, C8, C12, C16, C20 and C24. To gain insight into the transcriptome dynamic changes of maize seedlings in response to low temperature stress more subtly, we arranged to gather samples which named as C1, C2 and C3 every hour during the first 0 ~ 4 h of low temperature treatment. Furthermore, we wanted to observe the adaptability of inbred line B73 under long-term low temperature and taken samples at the fourth day under low temperature stress every four hours, which denoted as C72, C76, C80, C84, C88, C92 and C96 (Fig. 1).

Three biological replicates were included at each time points. In total 69 libraries were constructed in 23 time points; however, one library was discarded due to poor data quality. A total of 70 million reads were obtained from these 68 libraries. After filtering the initial data, we obtained Clean Reads and each sample retained approximately 10.64GB of valid data. In the present study, the Q20 value of Clean Reads for all samples was above 90%, and the value of Q30 was around 85%, indicating that the samples of mRNA were well preserved, and the transcriptome data were relatively reliable. The total mapping reads, and uniquely mapping reads are summarized in Table 1.

Table 1
Summary of RNA sequencing and mapping using the Maize B73 genome as the reference.

Sample	Total Clean Reads (Mb)	Total Clean Bases (Gb)	Clean Reads Q30(%)	Total Mapping (%)	Uniquely Mapping (%)
N0_1	71.44	10.72	84.16	88.39	55.23
N0_2	71.45	10.72	85.27	88.41	56.54
N0_3	71.46	10.72	84.91	88.83	56.34
N4_1	71.46	10.72	85.47	89.48	59.16
N4_2	71.43	10.71	85.34	89.2	58.46
N4_3	69.91	10.49	85.98	90.23	61.21
N8_1	69.93	10.49	86.3	89.94	61.11
N8_2	71.65	10.75	85.23	89.72	59.45
N8_3	69.9	10.48	84.73	89.04	57.92
N12_1	69.91	10.49	85.47	89.53	57.69
N12_2	71.65	10.75	85.27	89.23	58.71
N12_3	69.92	10.49	85.74	89.5	58.1
N16_1	69.91	10.49	85.27	89.67	58.65
N16_2	71.66	10.75	84.79	89.95	57.44
N16_3	69.93	10.49	86.08	89.43	60.19
N20_1	69.92	10.49	85.98	89.43	61.7
N20_2	69.91	10.49	85.56	89.93	60.53
N20_3	71.69	10.75	86.54	89.86	62.22
C0_1	71.66	10.75	85.69	90.88	63.46
C0_2	71.66	10.75	85.75	90.54	62.18
C0_3	71.65	10.75	85.23	90.86	62.75
C1_1	71.47	10.72	85.3	90.39	61.19
C1_2	71.45	10.72	85.74	90.09	61.52

Columns represent number of total clean reads, number of total clean bases, the percent of clean read Q30, the percent of total mapping and the percent of uniquely mapping. N0 ~ N20 represent samples under natural conditions before low temperature treatment. C0 ~ C96 represent samples under low temperature treatment. The numerical values 1, 2, 3 indicate the different biological replicates. The C72 datasets consisted of two replicates, all other datasets correspond to three replicates.

Sample	Total Clean Reads (Mb)	Total Clean Bases (Gb)	Clean Reads Q30(%)	Total Mapping (%)	Uniquely Mapping (%)
C1_3	71.45	10.72	85.78	89.59	61.22
C2_1	71.44	10.72	85.83	89.56	62.13
C2_2	71.6	10.74	86.25	89.71	62.34
C2_3	71.48	10.72	86.07	89.93	61.12
C3_1	71.49	10.72	86.08	90.15	62.58
C3_2	71.45	10.72	85.62	90.09	61.82
C3_3	69.91	10.49	84.94	90.03	60.69
C4_1	71.65	10.75	84.88	89.15	60.52
C4_2	71.66	10.75	85.34	89.51	61.34
C4_3	71.68	10.75	85.53	88.39	60.58
C8_1	71.63	10.75	84.84	88.33	60.37
C8_2	71.67	10.75	86.48	89.83	63.88
C8_3	69.92	10.49	86.41	90.14	63.83
C12_1	69.94	10.49	85.22	90.27	62.1
C12_2	71.44	10.72	85.78	90.61	62.41
C12_3	69.68	10.45	86.07	89.61	62.78
C16_1	71.42	10.71	86.05	89.43	61.53
C16_2	71.41	10.71	85.81	89.5	60.23
C16_3	71.44	10.72	85.84	90.37	62.17
C20_1	71.44	10.72	85.77	90.87	62.34
C20_2	71.46	10.72	86.18	91.09	62.47
C20_3	71.43	10.71	85.33	90	60.47
C24_1	71.68	10.75	86.34	91.3	62.67
C24_2	69.95	10.49	87.05	90.68	62.7

Columns represent number of total clean reads, number of total clean bases, the percent of clean read Q30, the percent of total mapping and the percent of uniquely mapping. N0 ~ N20 represent samples under natural conditions before low temperature treatment. C0 ~ C96 represent samples under low temperature treatment. The numerical values 1, 2, 3 indicate the different biological replicates. The C72 datasets consisted of two replicates, all other datasets correspond to three replicates.

Sample	Total Clean Reads (Mb)	Total Clean Bases (Gb)	Clean Reads Q30(%)	Total Mapping (%)	Uniquely Mapping (%)
C24_3	69.93	10.49	85.98	90.65	61.41
C72_2	69.93	10.49	86.22	90.91	63.38
C72_3	69.96	10.49	87.19	90.76	64.2
C76_1	71.7	10.75	87.06	91.01	63.76
C76_2	69.94	10.49	86.66	90.95	63
C76_3	69.95	10.49	86.73	91.63	64.05
C80_1	71.68	10.75	85.97	90.86	62.89
C80_2	69.95	10.49	86.93	91.91	65.27
C80_3	69.92	10.49	85.64	91.56	62.86
C84_1	69.93	10.49	86.33	92.03	64.16
C84_2	71.69	10.75	86.47	91.96	64.44
C84_3	71.68	10.75	86.03	91.22	63.24
C88_1	71.67	10.75	86.09	91.34	63.31
C88_2	71.64	10.75	85.69	90.47	63.17
C88_3	69.92	10.49	86.67	90.14	64.46
C92_1	69.91	10.49	86.5	90.39	63.76
C92_2	71.65	10.75	86.31	89.98	63.45
C92_3	71.66	10.75	86.42	90.88	64.31
C96_1	71.66	10.75	86.59	90.52	64.07
C96_2	69.92	10.49	86.81	90.81	64.87
C96_3	71.66	10.75	86.5	90.64	64.82

Columns represent number of total clean reads, number of total clean bases, the percent of clean read Q30, the percent of total mapping and the percent of uniquely mapping. N0 ~ N20 represent samples under natural conditions before low temperature treatment. C0 ~ C96 represent samples under low temperature treatment. The numerical values 1, 2, 3 indicate the different biological replicates. The C72 datasets consisted of two replicates, all other datasets correspond to three replicates.

3.2 RNA-Seq samples correlation analysis and principal component analysis

The quality of the transcriptome data has been tested and then we intended to verify the correlation among three different biological replications of the same sample (Figure S1). The total Clean Reads were generated and then mapped to the maize B73 reference genome (Zea_mays. B73_RefGen_v4.45) using HISAT. An average ~ 61.69% of Reads were uniquely mapped and only the uniquely mapped reads were further used to calculate the normalized gene expression level as fragments per kilobase of transcript per million mapped reads (FPKM). Comparison of the three biological replicates showed that the expression values among them were highly correlated (average $R^2 \geq 0.8$). Hence, we took the average FPKM value of the three replicates as the expression level for the sample at each time point. To reduce the influence of transcription noise, we defined a gene as expressed if its FPKM value was ≥ 1. In total, 23014 genes including 1363 TFs were found to be expressed in at least one of the 23 samples (Table S1). In addition, the highly correlation coefficient between the group treated at low temperature for one day and the control group, while the low Pearson correlation coefficient between the group treated at low temperature for 4 days and the control group (Figure S1). These results indicated that a small of genes changed expression after experiencing cold stress instantaneously in maize B73. In the case of continuous low temperature, there will be many genes differentially expressed in maize, to improve the survival ability of maize under continuous low temperature.

To provide an overview of the transcriptomic landscape and reduce the dimensions of the large datasets, a principal component analysis (PCA) was performed based on the princomp function in the R environment (Fig. 2). PC1 and PC2 were able to explain 30.9% and 17.1% of transcriptome variation respectively. PCA analysis of samples before and after low temperature treatment showed high similarity among the three replicates of each sample, indicating high quality of data set. In addition, 23 time point samples can be divided into 4 different stages. In stage □, the control group samples were clustered together, which was mainly explained by PC2, indicating that PC2 may be related to the regulation of circadian cycle. In the stage □, before 8 h low temperature treatment, C1, C2, C3, C4 and C8 could be explained by PC1, indicating that the transcriptome changes of maize inbred line B73 were not dramatic at this stage. Samples from C1 to C8 form the second cluster and represented the stage of early low temperature treatment. In the stage □, the transcriptome of all samples from 12h to 24h, including C12, C16, C20, C24, underwent dramatic changes at low temperature treatment. The samples between time points C12 and C24 formed third cluster and represented the stage of low temperature adaptation. In the stage □, all samples treated at low temperature for 3 days, including C72, C76, C80, C84, C88, C92 and C96, were clustered together, indicating that continuous low temperature treatment greatly changed the transcriptome of maize inbred line B73, and photoperiod had little influence on the transcriptome at this stage. Sample C72, C76, C80, C84, C88, C92 and C96 in the stage four could corresponds to the long-term low temperature stage.

3.3 Global transcriptome changes in maize seedings during low temperature treatment

The total mapped reads of all genes were used for differential expression analysis using DESeq [P-value (P) < 0.01] with gene expression levels calculated as FPKM. In the present study, we compared treatment groups (8℃ treatment) with control groups, which were at the same time points (Figure S2). A total of 22446 differentially expressed genes were detected in this study. We counted the number of differentially expressed genes (DEGs) between different groups in Table 2. In the early stage of low-temperature treatment (0–3 h), the number of DEGs was relatively small, there were only 2773 DEGs between C0 and C1. When the low temperature treatment lasted for a short time (4–8 h), the number of DEGs increased significantly. For example, there were 4694 DEGs between C4 and N4, and 7635 DEGs between C8 and N8. When the low temperature lasted for a long time (8–24 h), however, the number of DEGs increased significantly, and the expression of 77.09% DEGs decreased significantly (N20_C20). When the low temperature lasted for three days (72–96 h), there was no conspicuous change, but more genes showed a trend of high expression. The above results indicated that short-term low temperature treatment had little change in the transcriptome profile of maize, while continuous low temperature had a greater impact on maize seedlings.

Table 2
Number of genes exhibiting changes in expression in the B73 inbred line following low temperature induce.

Compare group	Up	Down	Total
N0_C0	3611	1302	4913
N4_C4	2915	1779	4694
N8_C8	4298	3337	7635
N12_C12	4768	4533	9301
N16_C16	4458	4804	9262
N20_C20	2964	9971	12935
C0_C24	2759	10748	13507
N0_C72	5824	6550	12374
N4_C76	5116	7439	12555
N8_C80	6460	5099	11559
N12_C84	7767	5503	13270
N16_C88	7423	5378	12801
N20_C92	5481	6802	12283
C0_C96	4258	6376	10634
C0_C1	1004	1769	2773
C0_C2	1384	1487	2871
C0_C3	1033	1848	2881

Up represents the number of up-regulated genes, Down represents the number of down-regulated genes.

We further performed the KEGG pathways enrichment analysis on the DEGs at each time point (Fig. 3). At the early stage of low temperature, down-regulated DEGs were significantly enriched in pathways of Glutathione metabolism, Valine leucine and isoleucine degradation, ABC transporters and Glycerophospholipid metabolism and inositol phosphate metabolism (Fig. 3A). Notably, from C8 to C96, almost down-regulated DEGs were significantly enriched in Photosynthesis. At the adaptation stage, down-regulated DEGs were enriched in Protein processing in endoplasmic reticulum, Glycosylphosphatidylinositol (GPI)-anchor biosynthesis, Nucleocytoplasmic transport, Terpenoid backbone biosynthesis, and Purine metabolism pathways. At the long-term stage of low temperature, down-regulated DEGs were specific enrich in carbon metabolism pathways. Meanwhile, genes involved in Plant-pathogen interaction and MAPK signaling pathways-plant were up-regulated in all surveyed time

points (Fig. 3B). Almost all the up-regulated DEGs of all the surveyed groups were enriched in Cysteine and methionine metabolism, except for C0 vs C2 comparison. At the early stage, genes related in phenylpropanoid biosynthesis and biosynthesis of amino acids were significantly up-regulated. At the adaptation stage, genes related in inositol phosphate metabolism, Glycerolipid metabolism were specifically upregulated. Finally, genes related to Proteasome and mRNA surveillance pathway were specifically upregulated at the long-term stage.

3.4 Time series analysis of all samples

Genes sharing similar expression profiles are commonly implicated in relevant biological functions or metabolic pathways. To better decipher the transcriptomic profiles in maize under cold stress, 23014 expressed genes were divided into 16 different cluster (Cluster1-Cluster16) according to their expression profiles in surveyed samples (Table S2). Each Cluster has a different number of genes, for example, there are 3005 genes in Cluster 9 but only 525 genes in Cluster 6 (Table 3). Furthermore, the gene expression profiles were distinct among the different clusters (Fig. 4 and Figure S3). Genes in Cluster1, Cluster2 and Cluster3 were highly expressed under normal conditions, but the expression level was decreased after chilling treatment. Genes in cluster 4 were higher expressed in the stage of early low temperature treatments (C1 – C8). Genes in Cluster 4 may be related to the rapid early response of maize to low temperature stress. Genes in cluster 7 were higher expressed at the adaptation stage of low-temperature treatment (C12 – C24). Genes in Cluster 8 and 9 the genes were specifically highly expressed in samples which were treated at low temperature for three days (C72- C96), suggesting that high expression of these genes may be related to the adaptability of maize to long-term low temperature. In addition, the expression of these genes in Cluster10 and Cluster11 decreased significantly with the continuation under low temperature treatment and was at the lowest after chilling treatment for three days. Additionally, genes in Cluster 5 were highly expressed in samples C3-C16, and genes in Cluster 6 were highly expressed in sample C16, implying genes in these clusters may play roles in chilling stress response. Genes in Cluster 16 were highly expressed in samples which were treated at low temperature after 12 hours (C12-C96), strongly impressing the important roles of those genes in maize cold stress response.

Table 3
Gene number and transcription factors number in each cluster

Clusters	Total Gene Number	Transcription Factors number	Ratio (%)
Cluster1	1137	67	5.89
Cluster2	1225	65	5.31
Cluster3	966	41	4.24
Cluster4	642	101	15.73
Cluster5	796	106	13.32
Cluster6	525	54	10.29
Cluster7	904	88	9.73
Cluster8	1626	117	7.2
Cluster9	3005	140	4.66
Cluster10	2794	140	5.01
Cluster11	1632	64	3.92
Cluster12	1956	85	4.35
Cluster13	1361	54	3.97
Cluster14	1194	47	3.94
Cluster15	1971	92	4.67
Cluster16	1280	102	7.97

3.5 Genes expressed under natural conditions

The stage under natural stage (N0-C0) is best represented by 3328 expressed genes in Cluster1 to Cluster3. The Cluster1 (1137 genes) contains a set of genes related to photosynthesis process and chlorophyll metabolism (Fig. 5). The Cluster2 (1225 genes) and Cluster3 (966 genes) corresponds to genes had the highest expression levels at N12 or N20 respectively and then appeared to be low or not expressed at other time points. KEGG analysis showed that genes in cluster 1 were enriched in photosynthesis, photosynthesis-antenna proteins and Porphyrin and chlorophyll metabolism. Genes in cluster 2 and 3 showed similar expression pattern, and genes in the two clusters enriched in the same pathways, including Photosynthesis-antenna proteins, cutin, suberine and wax biosynthesis, Phenylpropanoid biosynthesis, Flavonoid biosynthesis, and Fatty acid elongation (Fig. 5). Therefore, these genes might be involved in the normal growth and development of maize.

3.6 Genes expressed in the early stage of low temperature treatment

The early stage of low temperature treatment is best represented by 642 genes in Cluster 4. These genes are significantly enriched in pathways associated with MAPK signaling, plant-pathogen interaction, Benzoxazinoid biosynthesis, Glycerophospholipid metabolism, alpha-Linolenic acid metabolism, Phosphatidylinositol signaling system and Plant hormone signal transduction (Fig. 5). Notably, four marker genes, the homologs of *AtZAT10* (*Zm00001d013208*), *AtCBF3* (*Zm00001d006170*), *AtERF6* (*Zm00001d029910*), *AtCBF1* (*Zm00001d002618*) are also members of this cluster (Fig. 6).

3.7 Genes expressed in low temperature adaptation stage

The stage of low temperature adaptation (C12-C24) is best represented by 904 expressed genes in Cluster 7. Genes in cluster 7 were enriched in plant hormone signal transduction and sulfur metabolism (Fig. 5). Four marker genes, the homologs of *AtRAV2* (*Zm00001d03782*), *AtCOL5* (*Zm00001d024200*), *AtAPD1* (*Zm00001d016535*), *AtCOL5* (*Zm00001d049347*) are members of this cluster (Fig. 6).

3.8 Genes expressed in long-term low temperature stage

The stage of long-term low temperature (C72-C96) is best represented by 4631 genes, in Cluster8 and Cluster9. The genes in Cluster8 (1626 genes) showed peak expression at C88, whereas genes in Cluster9 (3005 genes) were highly expressed from C72 to C96. Although genes in cluster 8 and 9 showed similar expression pattern, their enriched KEGG pathways were largely distinct. Genes in cluster8 were enriched in Amino sugar and nucleotide sugar metabolism, beta-Alanine metabolism and Fatty acid degradation. Genes in cluster9 were enriched in pathways related to proteasome, spliceosome, protein processing in endoplasmic reticulum, RNA transport, gluthione metabolism, SNARE interactions in vesicular transport, glycolysis/gluconeogenesis and basal transcription factors (Fig. 5). Four marker genes, the homologs of *OsAsr1* (*Zm00001d023529*), *OsSAP8* (*Zm00001d015842*), *OsLti6b* (*Zm00001d024778*), *AtCOR47* (*Zm00001d051420*) are members of cluster 9 (Fig. 6). Finally, in cluster 16, genes were highly expressed in both stage of low temperature adaptation and long-term low temperature stress. There were 1280 genes in Cluster 16. Genes in Cluster16 were enriched in pathways related to phenylpropanoid biosynthesis, MAPK signaling pathway, plant-pathogen interaction and plant hormone signal transduction (Fig. 5).

3.9 TFs in different gene Clusters

Transcription factors function important roles in plant cold stress response. TFs in different gene clusters were identified in the results (Table 3). In Cluster 4, which genes highly expressed in the early stage of low temperature treatment (C1-C8), as many as 15.73% genes were TFs. Different from the early stage of low temperature stress, there were fewer genes encoding transcription factors (9.7%) in cluster 7, in which genes highly expressed in stage of low temperature adaptation (C12-C24). In Cluster 8 and 9, in which genes highly expressed in long-term low temperature stress, 7.22% and 4.66% TFs were

identified. In cluster 16, genes were highly expressed in both stage of low temperature adaptation and long-term low temperature stress, 7.97% genes were TFs. In contrast, Clusters 1, 2, and 3, which primarily contained genes highly expressed during the natural stage (N0–C0), exhibited a relative lower transcription factor proportions, at only 5.89%, 5.31%, and 4.24%, respectively. In addition, a total of 49 transcription factor (TF) families were identified across the five low-temperature stress responsive clusters (Clusters 4, 7, 8, 9, and 16) (Table S3). The most prominently represented families included AP2-EREBP, MYB, WRKY, bHLH, NAC, and GRAS, which together accounted for the majority of TFs in all these five clusters. In addition, several families showed cluster-specific. For example, HSFs were not found in cluster 4 and 7, but was almost entirely confined to Cluster 9 (12 members), with only one occurrence in Cluster 8 and 9. EILs were exclusively found in Cluster 16 (4 members), and C2C2-CO-like appeared only in Cluster 7 (4 members). Other families such as ARF, bZIP, C3H, and C2H2 were widely distributed but at lower counts (1–6 members per cluster).

3.10 ZmADC1 and ZmADC2 were induced by low temperature, and their tissue expression patterns, and subcellular localization were analyzed

In above results, genes in cluster 4 and 16 may important in response to cold stress in maize. Intriguingly, two genes, *Zm00001d045470* and *Zm00001d051194*, which were homologous genes and annotation as the arginine decarboxylase, were identified from cluster 16 and 4, respectively. Arginine decarboxylase were important to the putrescine biosynthesis, and putrescine accumulation was essential for proper cold accumulation [19]. The transcriptome analysis results also showed that the expression levels of two key genes were up regulated under low temperature stress. As shown in Fig. 7 (a), under low temperature condition, the expression of *Zm00001d045470* continued to increase, while the expression of *Zm00001d051194* slowly decreased after a short increase.

Previous reports showed that genes encoding arginine decarboxylase belong to alanine racemase (AR) family and harbored the Orn_Arg_deC_N (PF02784.15) domain. A genome-wide protein domain analysis identified five additional maize genes encoding proteins that also harbor the Orn_Arg_deC_N domain (PF02784.15) and belong to the AR family (Table 4). Additionally, a phylogenetic tree was constructed with these seven maize genes and other reported genes belong to AR family from rice and Arabidopsis. (Fig. 7b). The phylogenetic tree is divided into three branches, all belonging to the alanine racemase (AR) family, namely diamino-malonic acid decarboxylase (DapDC), ornithine decarboxylase (ODC) and ADC. Phylogenetic relationship showed that Zm00001d045470(cluster16) and Zm00001d051194（cluster4 ）have high homology with OsADC1 and OsADC2 of rice, so we named these two genes *ZmADC1* and *ZmADC2*, respectively.

Table 4
Physicochemical Properties of members in maize Arginine decarboxylase gene family

Gene ID	Location	Group	Gene Length (bp)	Protein Length (aa)	Mw	PI
Zm00001d007481	Chr2: 232695558–232696814		1257	418	44216.12	6.26
Zm00001d051194	Chr4: 148148821–148150740		1920	630	70756.67	5.18
Zm00001d021321	Chr7: 148806602–148807834		1233	410	43509.61	5.63
Zm00001d022593	Chr7: 180714727–180716026		1300	412	44305.43	5.96
Zm00001d045470	Chr9: 23397355–23399439		2085	694	73506.53	5.06
Zm00001d023429	Chr10: 5402577–5411941		9365	488	53291.13	8.27
Zm00001d000153	Ctg75: 228571–239357		10787	344	53090	7.12
Mw means Molecular Weight; PI means Isoelectric Point						

Tissue-specific expression patterns of *ZmADC1* and *ZmADC2* were also investigated.

As shown in Fig. 7 (d), the relative expression of *ZmADC1* in roots, stems, bracts, male ears and grains was low on average with little difference, and in leaves (V4) was relatively high, while the expression level in pollen was significantly higher than that in other tissues. *ZmADC2* was highly expressed in stems, leaves (V4), bracts (R1) and pollen of maize, but relatively low in primary roots, air roots, secondary roots and grains. To further understand the expression locations of ZmADC1 and ZmADC2 in cells, we constructed fusion expression vectors pCAMBIA1305-35S-*ZmADC1*-GFP and pCAMBIA1305-35S-*ZmADC2*-GFP and performed transient expression in maize protoplasts. It can be seen from Fig. 7 (c) that ZmADC1 is mainly located in the nucleus and cytoplasm, while ZmADC2 is mainly located in the endoplasmic reticulum.

3.11 Overexpression of *ZmADC1* and *ZmADC2* can improve the low temperature tolerance of rice

To further investigate whether *ZmADC1* and *ZmADC2* could be responsive to cold stress, *ZmADC1* and *ZmADC2* were heterologously expressed in rice (variety 9311). The expression of *ZmADC1* and *ZmADC2* in rice was determined by RT-qPCR, and the results were shown in Fig. 8 (a). According to the level of gene expression, we finally chose $9311^{ZmADC1\#OE4}$, $9311^{ZmADC1\#OE5}$ and $9311^{ZmADC2\#OE8}$, $9311^{ZmADC2\#OE9}$

for follow-up research. Next, we selected three-leaf stage hydroponic rice seedlings to be treated with low temperature at 8 ℃ for 4 days, and then grew normally for 7 days. The phenotypes of wild type 9311, *ZmADC1* transgenic lines and *ZmADC2* transgenic lines under low temperature stress are shown in Fig. 8 (b). Through the statistics of rice survival rate, we found that wild type 9311 basically died. $9311^{ZmADC1\#OE4}$, $9311^{ZmADC1\#OE5}$ and $9311^{ZmADC2\#OE8}$, $9311^{ZmADC2\#OE9}$ all had a survival rate of more than 50%, and the survival rate was significantly higher than wild type. The statistical results of survival rate are shown in Fig. 8 (c). We made statistics on putrescine, MDA and CAT of rice under low temperature stress. The change of putrescine content under low temperature stress is shown in Fig. 8 (d) and Fig. 8 (f). Under the normal growth condition of rice seedlings, the putrescine content of *ZmADC1* and *ZmADC2* transgenic lines was higher than wild type, and the putrescine content of transgenic lines was still higher than wild type after low temperature stress. The detection results of MDA and CAT were shown in Fig. 8 (e) and Fig. 8 (g). The increase of MDA content of *ZmADC1* and *ZmADC2* transgenic rice lines after low temperature treatment was lower than wild type, while the MDA content of *ZmADC1* transgenic lines was lower than *ZmADC2* transgenic lines. CAT content increased rapidly after 24 h of low temperature treatment and decreased at 48 h, but the CAT content of transgenic lines was generally higher than wild type.

4. Discussion

Maize originated from tropical and subtropical regions and is inherently sensitive to chilling stress [15, 29]. Chilling stress threatens maize throughout the whole growth stage. But in actual agricultural production, cold injury is more severe during the seed germination and seedling stages [23]. Low-temperature chilling injury in early spring has a significant adverse effect on both the seed germination rate and the vitality of seedling. Under low temperature, diverse physiological and biochemical processes was damaged, resulting to growth inhibition, or even death of the seedlings[30]. Thus, deciphering the transcriptional regulatory pathway and key regulatory genes of maize seedlings under chilling stress is meaningful. At the present study, a time-series transcriptomic analysis was investigated of maize seedlings at the three-leaf stages under chilling stress at 16 time points (Fig. 2). Dynamic gene responses of early low temperature (chilling stress for 1 h, 2 h, 3 h, 4 h), low temperature adaptation (chilling stress for 8 h, 12 h, 16 h, 20 h, and 24 h), and long-term low temperature (chilling stress for 72 h, 76 h, 80 h, 84 h, 88 h, 92 h, and 96 h) were investigated. As the time of chilling stress lasted, more DEGs were identified (Table 2), short-term low temperature had little effect on the maize transcriptome, whereas continuous low temperature, particularly after 20 h of stress, had a greater impact on seedlings. Chilling-sensitive plants such as maize, showed photosynthetic efficiency decreased under low temperature [31]. According the functional annotation of DEGs in different time points, down regulated genes were enriched in photosynthesis and light harvesting, especially in low temperature adaptation and long-term low temperature stage (Fig. 3). Mitogen-activated protein kinase (MAPK) cascades have also been reported to play vital roles in plant cold signaling [32]. In our results, the MAPK signaling pathway emerged as a commonly enriched category among upregulated DEGs throughout all low-temperature treatment time points. Phenylpropanoid metabolism contributes to plant development and

plant-environment interactions [33]. Phenylpropanoid metabolism was also ubiquitously enriched in upregulated DEGs across almost all low-temperature treatment stages.

Clustering or co-expression is generally applied to group genes that have a similar expression pattern in multiple samples [34]. Genes showed similar expression profiles generally indicate to be functionally related or conserved [35]. At the present study, 23014 expressed genes were classified into 16 clusters (Fig. 4). Among these, five clusters, including cluster 4, 7, 8, 9 and 16, were identified as potentially representing distinct temporal phases of the low-temperature response. The early stage of low temperature treatment is best represented by Cluster4. CBFs were key TFs involving in cold stress response and quickly induced under low temperature [13]. Overexpression of AtCBF1 and AtCBF3 in Arabidopsis, tomato, potato and other plant species, could enhance the low temperature tolerance [36–38]. The homologous genes of *AtCBF1* (*Zm00001d002618*) and *AtCBF3* (*Zm00001d006170*) belong to cluster 4 (Fig. 6). Cluster4 was overrepresented by genes related to MAPK signaling pathway (Fig. 5). The stage of low temperature adaptation (C12-C24) is best represented by genes in Cluster7. Genes in cluster7 were enriched in plant hormone signal transduction. The stage of long-term low temperature (C72-C96) is best represented by Cluster8 and Cluster9. Genes in Cluster 16 were highly expressed in samples which were treated at low temperature after 12 hours, indicating their important roles of those genes in both the low temperature adaption and long-term low temperature stage. Genes in cluster 16 were enriched in MAPK signaling pathway and plant hormone signal transduction (Fig. 5). Collectively, these temporal expression patterns imply a dynamic and stage-specific transcriptional reprogramming during cold stress.

To date, numerous transcription factors (TFs) have been implicated in low-temperature stress responses. Members of the AP2/ERF family are considered pivotal transcriptional regulators in the plant response to cold stress, acting in concert with a suite of other TF families—notably WRKY, bHLH, bZIP, MYB, NAC, and C2H2—to collectively potentiate cold stress tolerance [39]. In the present study, the most prominently represented families included AP2-EREBP, MYB, WRKY, bHLH, NAC, and GRAS, accounting for the majority of TFs in all these five low-temperature stress responsive clusters (Cluster 4, 7, 8, 9, 16) (Table S3). TFs accounted for 15.73% of the genes in Cluster 4, 9.73% in Cluster 7, 7.2% in Cluster 8 and 4.66% in Cluster 9 (Table 3). These results indicating that more TFs are upregulated during the early phase of the chilling stress response in maize. Transcriptional regulatory events are rapidly mobilized to initiate downstream signaling cascades. As the acclimation phase proceeds, an increasing number of functional genes are recruited. Clusters 8 and 9 exhibited a lower proportion of TF-encoding genes but a higher prevalence of functional genes, many of which are specifically involved in metabolic processes.

Arginine decarboxylase (ADC) (EC 4.1.1.19) is responsible for Put biosynthesis, a diamine precursor of higher polyamines (PAs) [21]. PAs plays a vital role in plant stress response. Previous reports showed ADCs were important in plant cold stress response. ADCs could enhance cold or chilling stress resistance in Arabidopsis, *O.sativa*, tea, Poncirus trifoliata and so on [22, 40, 41]. *ZmADC1* from cluster16 and *ZmADC2* from cluster 4 showed relative near relationship with OsADC1 and OsADC2 (Fig. 7). Our results showed that the two maize genes could enhance rice chilling stress resistance and promote the

putrescines accumulation (Fig. 8). The results further highlighted the functional importance of genes belonging to clusters 4 and 16 in maize under chilling stress and also validated the accuracy of our data analysis. Our research provides an important theoretical basis for discovering chilling tolerance genes in maize.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors declare no conflict of interest.

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Author Contribution

Conceptualization, L.G. and W.S.; methodology, L.G., G.Z., and Z.W.; validation, G.Z., Z.W., S.Y., and X.H.; formal analysis, S.Y., Q.Z., X.H.; investigation, G.Z., Z.W., S.Y., X.H., Y.X., L.Z. and J.D.; resources, G.Z., and Z.W.; data curation, S.Y., X.H., Y.X., L. Z. and J.D.; writing—original draft preparation, G.Z., Z.W., and W.S.; writing—review and editing, L.G.; visualization, W.S.; supervision, X.H., Y.X., L.Z. and J.D.; funding acquisition, L.G.,W.S. and L.Z.. All authors have read and agreed to the published version of the manuscript. Guangqing Zhou and Zimeng Wang contribute equally to the manuscript.

Data Availability Statement

The raw sequencing data has been submitted to National Genomics Data Center at the Beijing Institute of Genomics, under submission ID subCRA086144.

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Figures

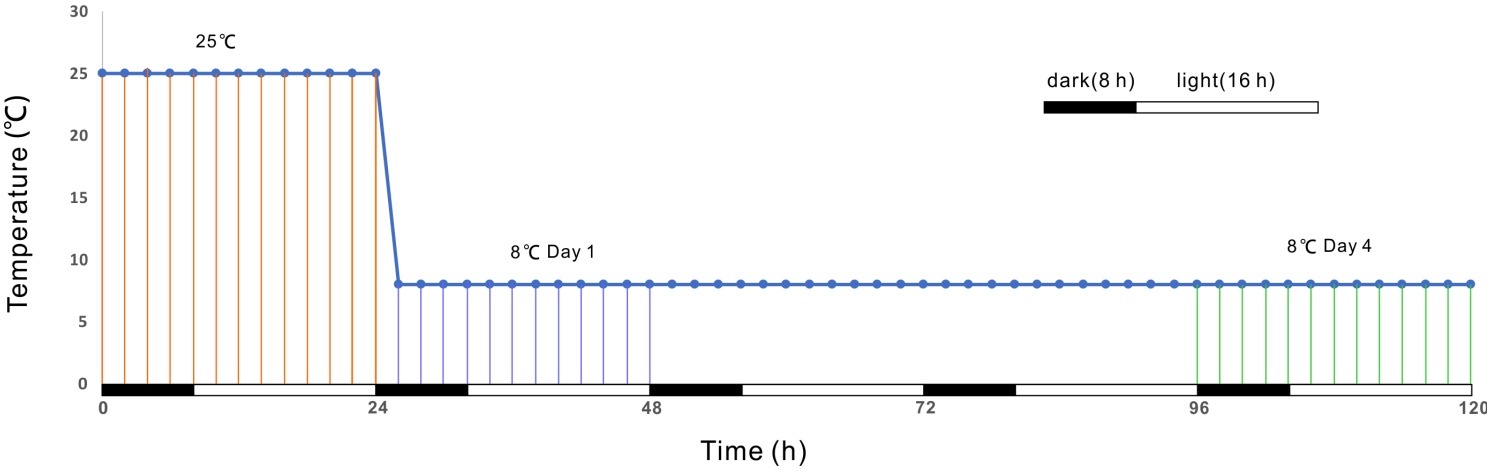


Figure 1

Chilling treatment to maize inbred line B73.

N represents seedlings were cultured in normal conditions (28 ℃, 16 h light/ 8 h dark), C represents seedlings were treated with low temperature (8 ℃, 16 h light/ 8 h dark).

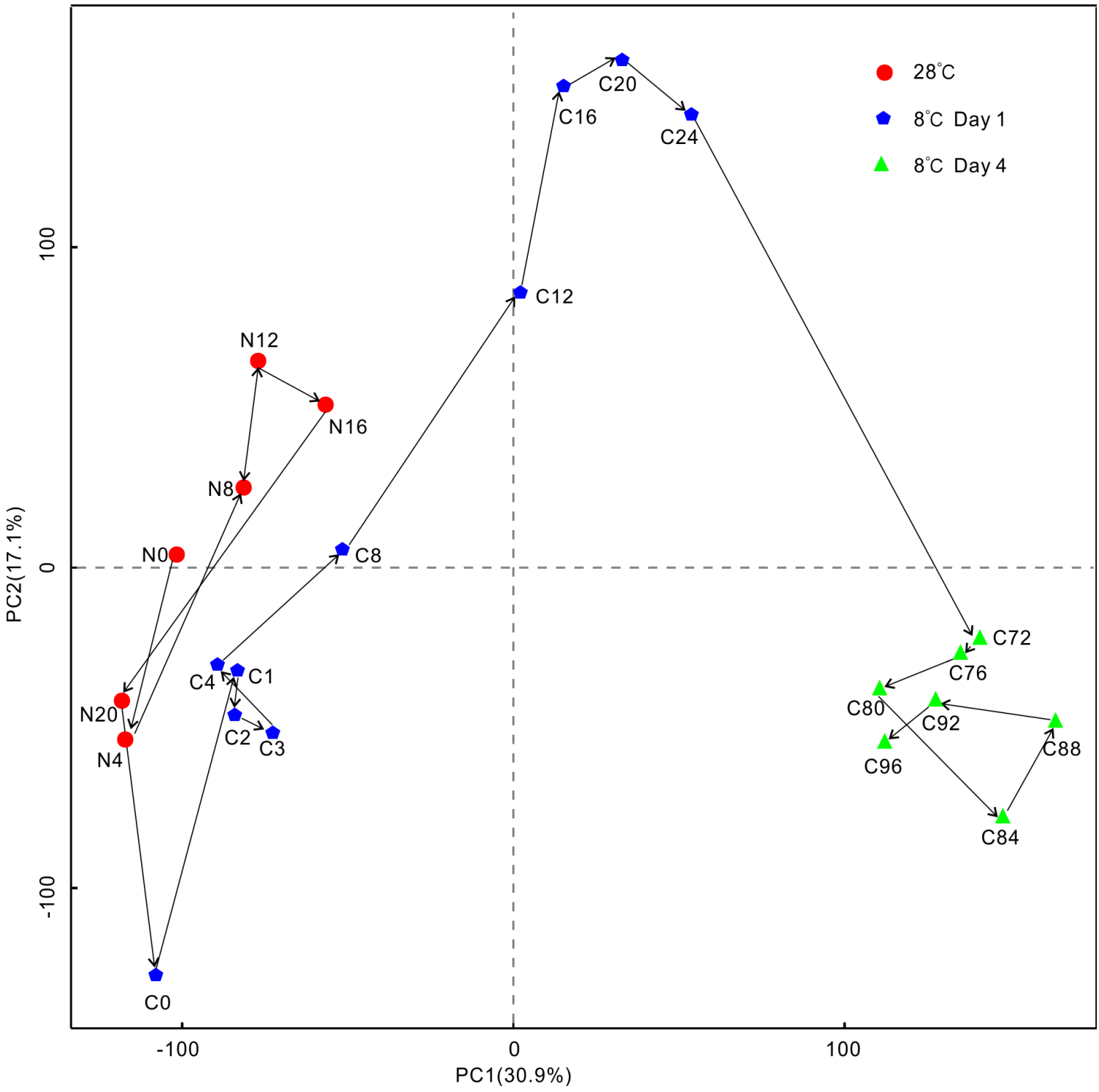


Figure 2

PCA analysis of all samples.

Evaluation of RNA-Seq data quality. Principle component analysis (PCA) factorial maps showing the largest components of variance. PCA was performed using the R function “prcomp” based on normalized read counts obtained from DESeq. PC1 (30.9% of the variance) and PC2 (17.1% of variance).

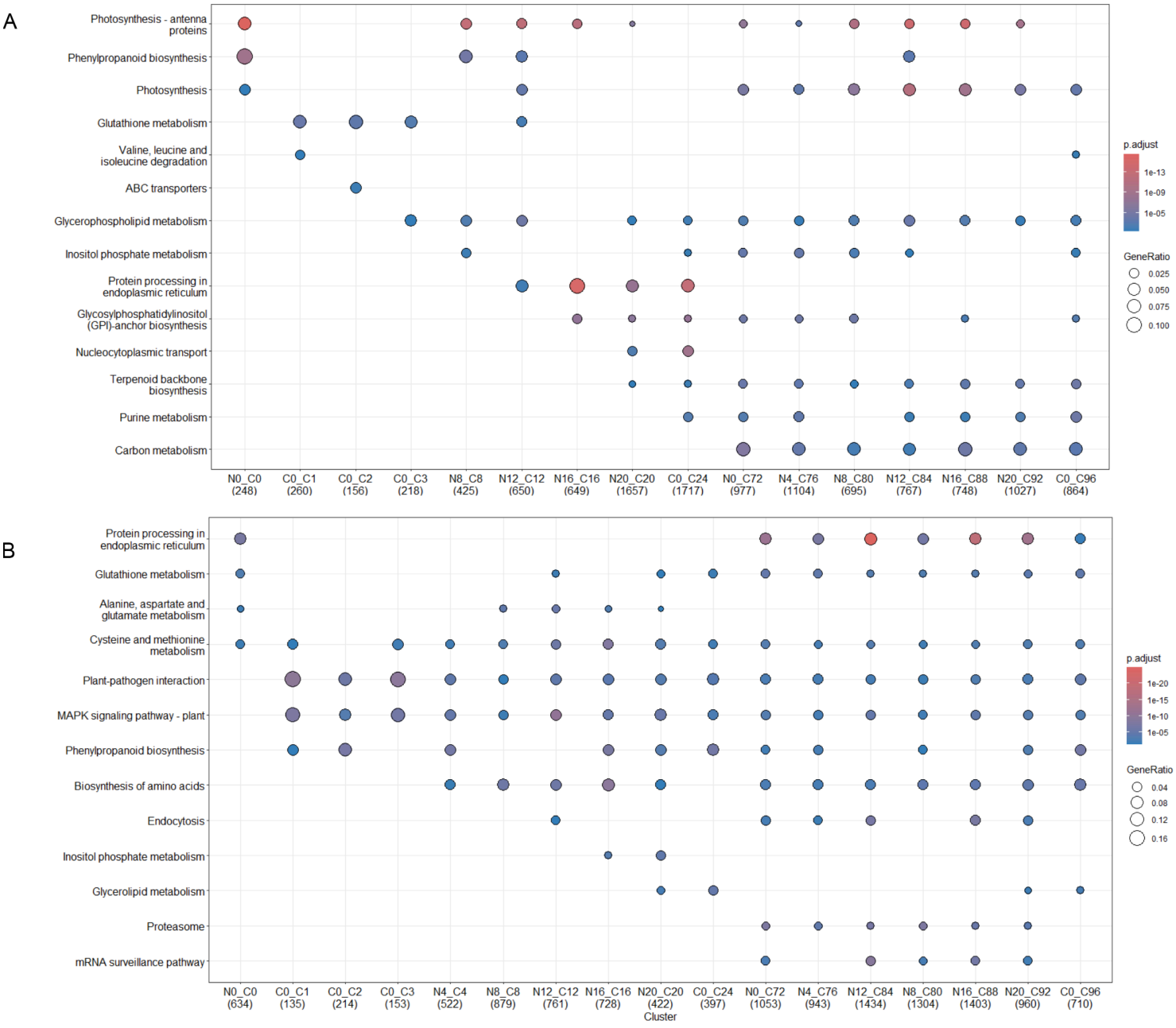


Figure 3

KEGG pathway enrichment analysis of differentially expressed down(A) and up (B) regulated genes in each chilling treated time points. The bubble plot displays the significantly enriched KEGG metabolic pathways for DEGs.

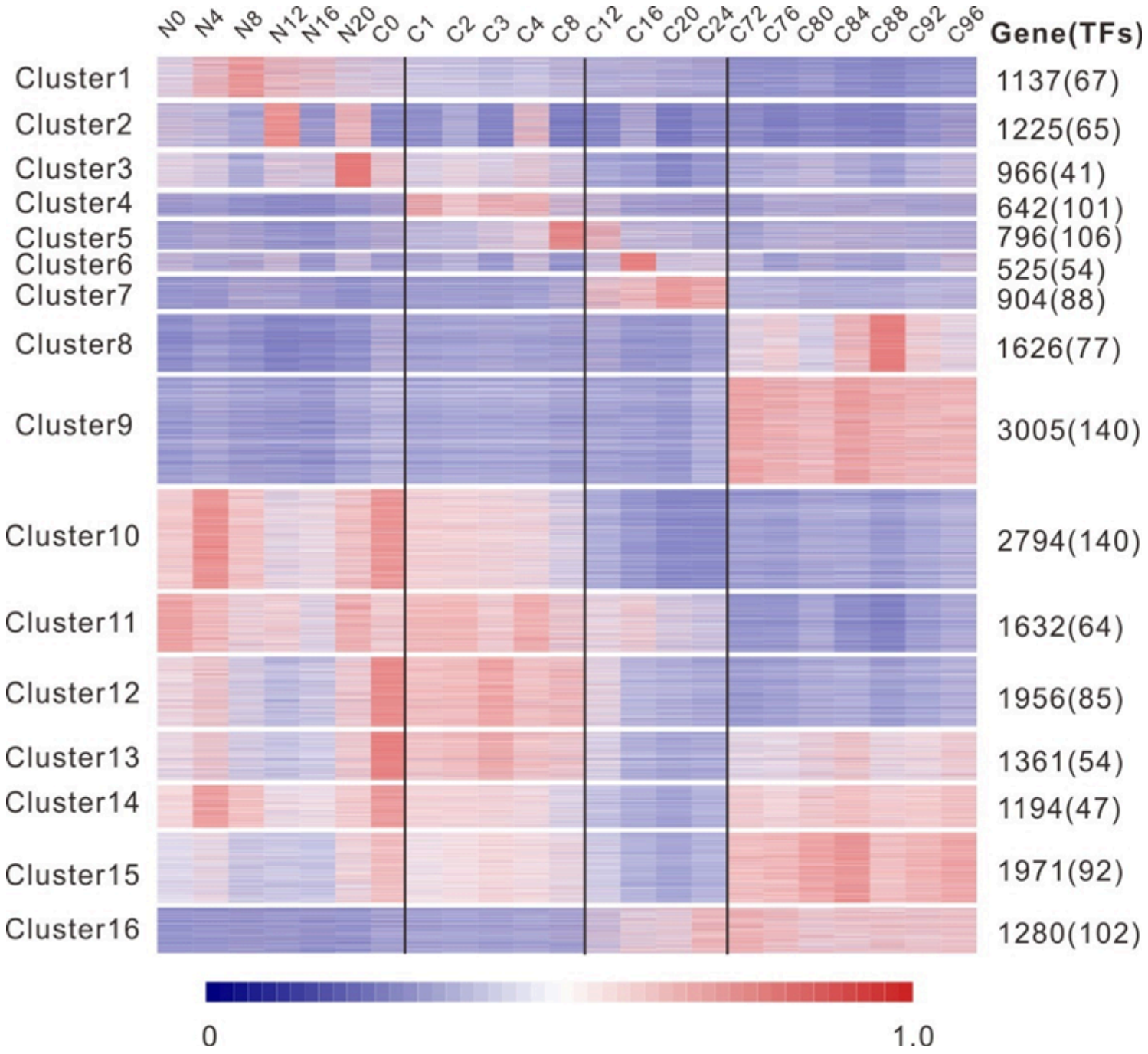


Figure 4

Hierarchical Clustering and heatmap of DEGs.

Expression patterns of genes in different clusters. For each gene, the FPKM value normalized by the maximum value of all FPKM values of the gene over all time points is shown. The number of genes and TFs in each cluster are shown on the right.



Figure 5

Functional transition over the time course.

KEGG pathway enrichment analysis of genes in different clusters.

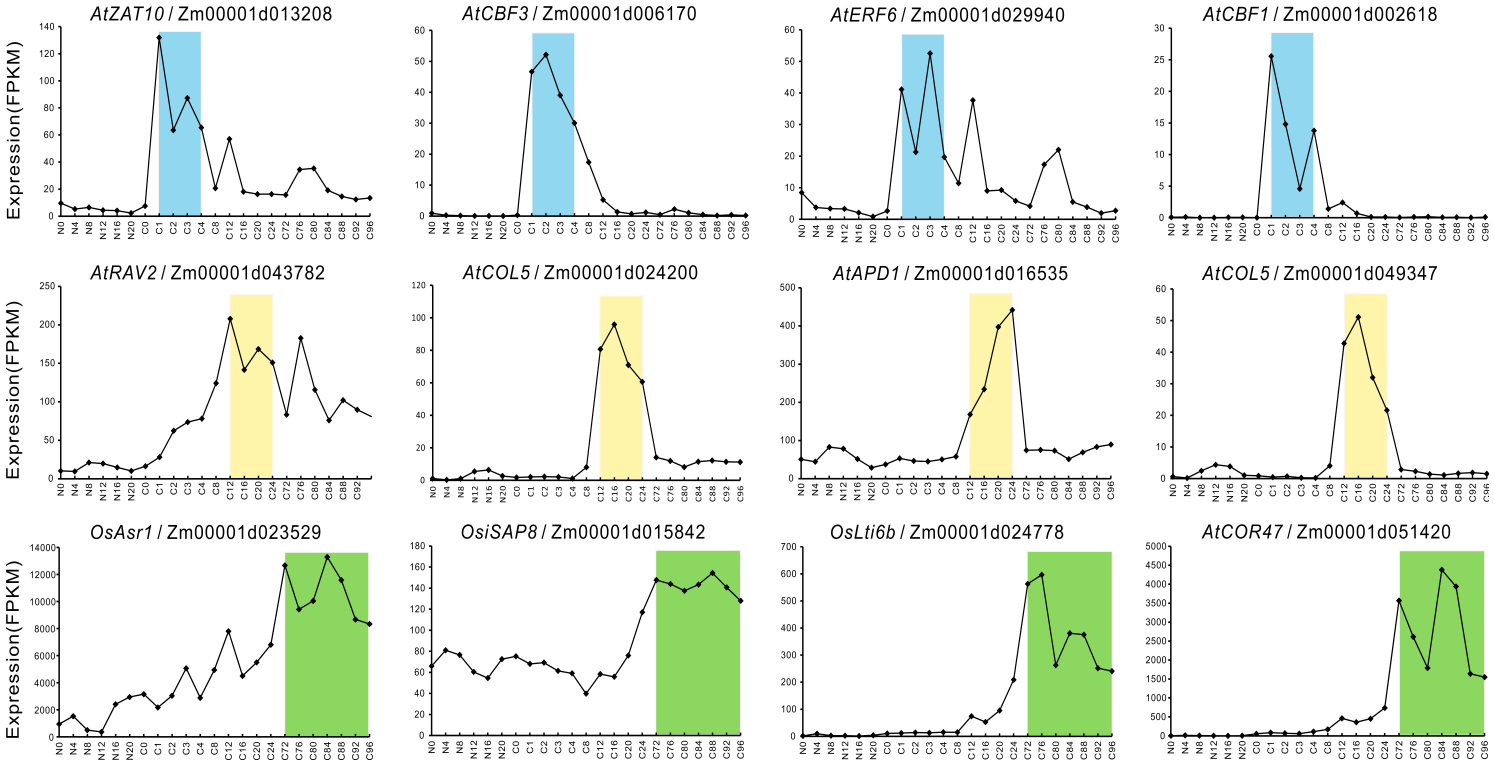


Figure 6

The marker genes mainly expressed in the early stage of low temperature, low temperature adaptation stage and long-term low temperature stage.

The time points belong to the stage of early low temperature treatment, low temperature adaptation, long-term low temperature shown in blue, yellow and green, respectively.

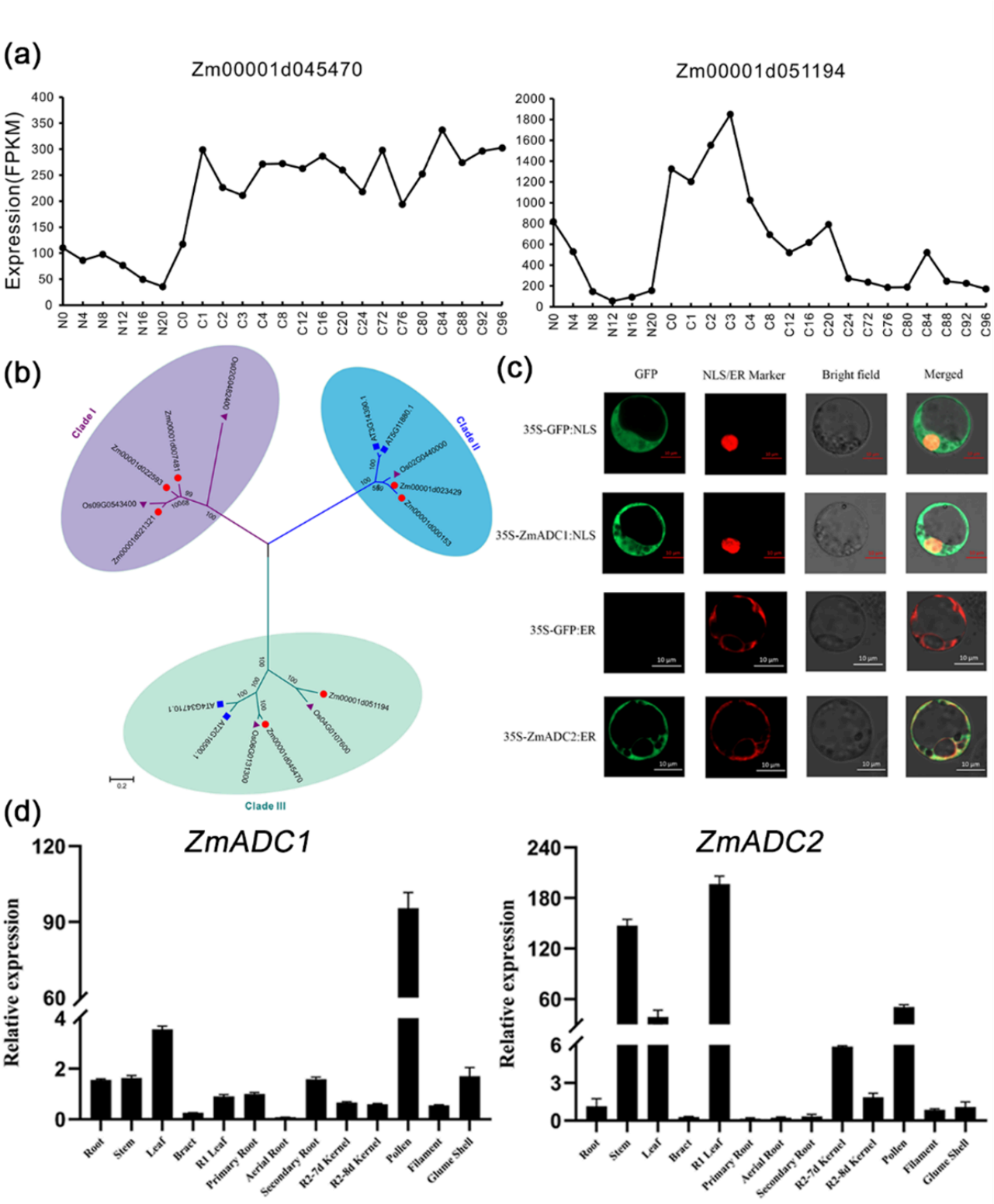


Figure 7

***ZmADC1* and *ZmADC2*were induced by low temperature, and their tissue expression patterns, and subcellular localization were analyzed.**

(a) The line graph depicting dynamic transcriptome-level changes of the *ZmADC1* and *ZmADC2*in maize under cold stress. (b) Phylogenetic analysis of amino acid sequences of maize arginine decarboxylase

with other known arginine decarboxylase of different species. (c) Confocal images of free GFP, ZmADC1-GFP and ZmADC2-GFP fusion protein driven by 35S promoter in maize protoplast, red fluorescence as a nuclear localization signal, Bar = 10 μm. (d) RT-qPCR analysis of *ZmADC1* and *ZmADC2* expression in different B73 tissues as indicated. The expression levels are normalized to the maize actin gene. Error bars represent ±SD from three technical replicates. Experiments were repeated three times using biological samples from different ears with similar results.

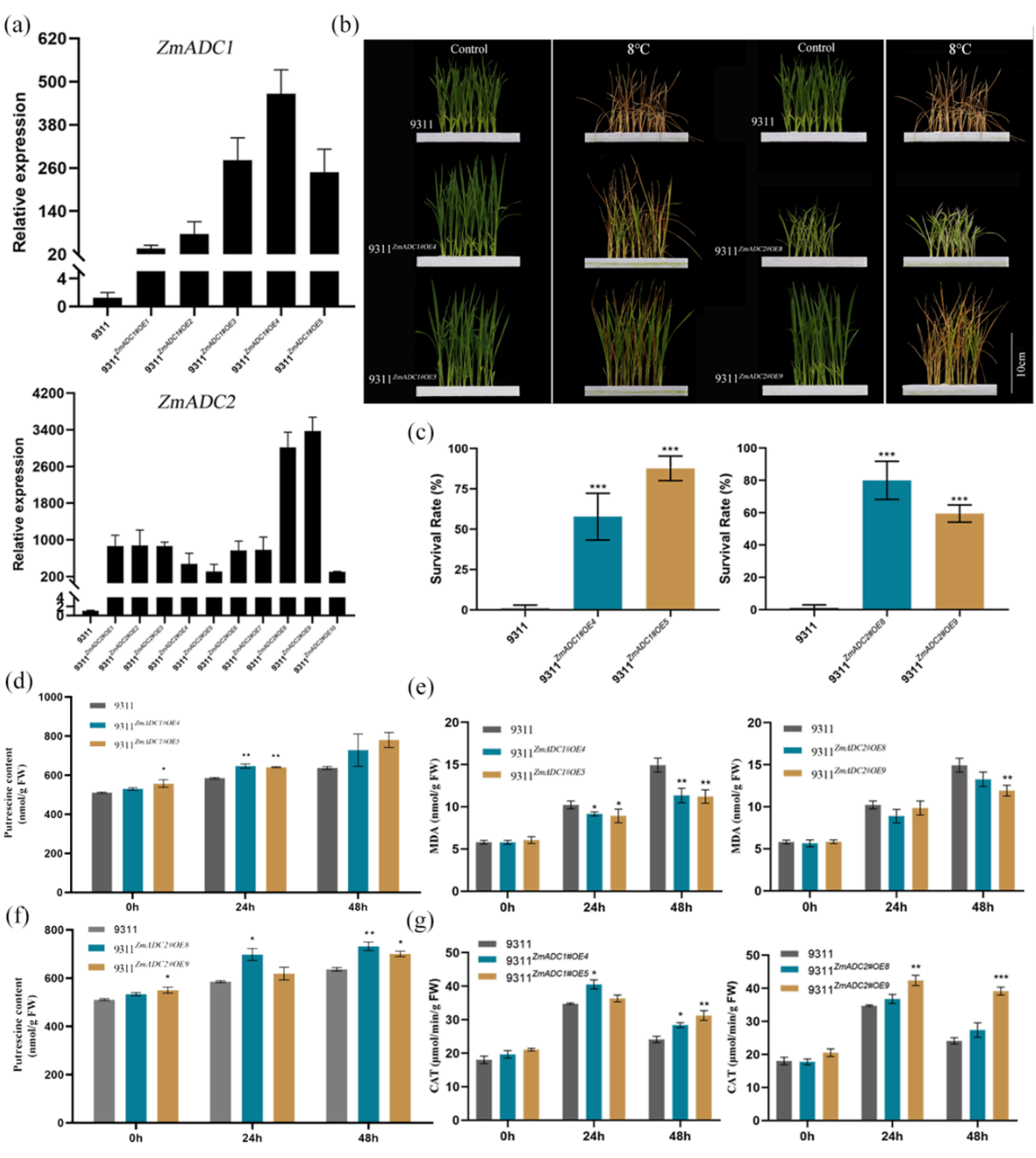


Figure 8

Legend not included with this version.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.docx
- SupplementaryTables.xlsx