



## Research article

# Transcriptome analysis of harvested bell peppers (*Capsicum annuum* L.) in response to cold stress

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## ABSTRACT

Bell peppers are valued for their plentiful vitamin C and nutritional content. Pepper fruits are susceptible to cold storage, which leads to chilling injury (CI); however, the crucial metabolic product and molecular basis response to cold stress have not been elucidated definitely yet. To comprehensively understand the gene regulation network and CI mechanisms in response to cold stress on a molecular level, we performed high-throughput RNA-Seq analysis to investigate genome-wide expression profiles in bell peppers at different storage temperatures (4 °C and 10 °C). A total of 61.55 Gb of clean data were produced; 3863 differentially expressed genes (DEGs) including 1669 up-regulated and 2194 down-regulated were annotated and classified between the CI group and control. Together, a total of 41 cold-induced transcription factor families comprising 250 transcription factors (TFs) were identified. Notably, numerous DEGs involved in biomembrane stability, dehydration and osmoregulation, and plant hormone signal transduction processes were discovered. The transcriptional level of 20 DEGs was verified by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Our results present transcriptome profiles of bell peppers in response to cold stress; the data obtained may be useful for the identification of key candidate genes and elucidation of the mechanisms underlying membrane damage during chilling injury.

## 1. Introduction

Bell peppers (*Capsicum annuum* L.) are widely consumed because of their high vitamin C content and health-enhancing effects. To maintain fruit quality and prevent peppers from decaying postharvest, low-temperature storage as an effective protective measure is extremely important. However, as a cold-sensitive fruit, bell peppers are susceptible to chilling injury (CI) at environmental temperatures of below 7 °C (Gonzálezaguilar et al., 2000). The typical symptoms caused by low temperature in peppers include surface pitting, the browning of calyxes and seed, and tissue discoloration, resulting in severe waste and economic loss (Hardenburg et al., 1986).

CI symptoms are distinct in different fruits and vegetables. For instance, the sarcocarp of peach and loquat undergoes lignification (Koushesh and Moradi, 2017); in Nanguo pears, the aroma ester weakens and pericarp browns (Shi et al., 2018). In banana, the vascular bundle undergoes browning in response to chilling injury (Luo et al., 2015). The most typical CI symptoms for peppers are surface pitting and calyxes browning. It has been confirmed in bell peppers that cold

stress accelerated membrane lipid peroxidation, and this process is usually accompanied by biomembrane structure damage and increased activity of membrane hydrolase (Wang et al., 2016). Studies have shown that the primary cause of plant chilling injury is physical phase of the biological membrane, then a variety of secondary reactions changes are triggered. One of the most crucial performance is the destruction of the cytomembrane structure and function. This change induce membrane lipid degradation, while also leading to the dehydration of cells (Kong et al., 2018). Accordingly, the cells secreting more organic and inorganic substances by self-osmotic adjustment to maintain water retention capacity. In addition, plant internal hormones level also respond to cold stress to heighten stress tolerance. It is reported that the content of abscisic acid increase significantly to protect plants from cell dehydration. Over the years, with the rapid development of high-throughput sequencing, it is possible to study the molecular mechanisms of cold response product during chilling injury in postharvest vegetables and fruits.

The development of next-generation sequencing (NGS) technologies makes it possible to monitor comprehensive gene expression changes in

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## Abbreviations

ABA	abscisic acid	KEGG	Kyoto Encyclopedia of Genes and Genomes
ABF	ABA-responsive element binding factor	KOG	euKaryotic Orthologous Groups
ALDH	aldehyde dehydrogenase	LOX	lipoxidase
ANOVA	one-way analysis of variance	LPLs	lysophospholipids
BR	brassinosteroid	LSD	least significant difference
CERS	ceramide synthetase	LTP	lipid transfer proteins
CI	chilling injury	NGS	next-generation sequencing
CK	cytokinin	NR	non-redundant
COG	Cluster of Orthologous Groups	PA	phosphatidic acid
DAG	diacylglycerol	PAO	polyamine oxidase
DEGs	differentially expressed genes	P5CDH	1-pyrroline-5-carboxylate dehydrogenase
DGD	digalactosyldiacylglycerol	P4HA	ornithine decarboxylase, prolyl 4-hydroxylase
ET	ethylene	PI/PC-TP	phosphatidylinositol/phosphatidylcholine transfer proteins
FPKM	fragments per kilobase of transcript per million fragments mapped	PLC	phospholipase C
GA	gibberellin	PLD	phospholipase D
FFAs	free fatty acids	PP2C	phosphatase 2C
Gala	alpha-galactosidase	Pfam	Protein family
GO	Gene Ontology	RH	relative humidity
JA	jasmonic acid	RT-qPCR	reverse transcription quantitative polymerase chain reaction
JAR1	jasmonic acid-amino synthetase	SA	salicylic acid
JAZ	jasmonate ZIM domain-containing protein	Tfs	transcription factors

plants more systematically. Further, high-throughput RNA-Seq has been used to detect global transcriptome dynamics and gene regulatory networks with higher sensitivity under multiple stress factors (Yao et al., 2018). To date, this high-efficiency technology has been applied to the study of numerous postharvest fruits such as banana, potato, and Nanguo pear. Previous studies have identified genes and TFs whose expression is regulated in response to cold stress; among these, DREB/CBF TFs are the most well-known TFs that positively correlate with cold by regulating cold-responsive genes (Fowler and Thomashow, 2002). A recent study of *Hevea brasiliensis* found numerous genes involved in abscisic acid metabolism and signaling in an abscisic acid-independent pathway, based on comparative transcriptome analysis (Cheng et al., 2018). Besides, Liu et al. (2018) found several membrane-related genes and biochemical processes that responded positively to cold stress in wild banana, by RNA-seq. Furthermore, new transcriptome and metabolome research in potato showed that the *ADCI*-associated putrescine pathway play a vital role in cold-acclimated freezing tolerance, putatively by promoting the expression of the CBF genes (Kou et al., 2018). However, because of plant specificity, differentially expressed genes exhibit different response patterns under cold stress. The early transcriptomics studies related to CI in pepper plants mainly focused on hot pepper leaves, and studies in harvested peppers are scarce. A recent transcriptomics study investigated the molecular effects in harvested peppers treated with jasmonate and 1-methylcyclopropene at 0 °C (Shin et al., 2017). However, genetic characterization of chilling injury due to cold-induced stress in postharvest bell peppers has not been performed to date.

In this study, a comprehensive transcriptome profiling analysis, along with physiological experiments on bell peppers at different storage temperatures (4 °C and 10 °C) were carried out to characterize the cold response mechanism of this fruit. Numerous cold-induced genes and TFs were found and their expression in the corresponding metabolism pathways described. This work aims to explore the overall gene network dynamics in bell peppers associated with chilling injury at the molecular level. The results of this study provide valuable data regarding the cold stress for postharvest fruits and vegetables. In addition, the work provides novel insights into the regulation of gene networks in pepper fruits.

## 2. Material and methods

### 2.1. Plant material and treatments

Bell peppers (*Capsicum annuum* L., cv 'fu ding lv xing') were obtained from a greenhouse in Jinzhou, Liaoning, China, and immediately transported to the laboratory. Pepper fruits with uniform size, similar maturity, and no damage were used for experiments. Pepper fruits were packaged with PVC film bags (0.03 mm) after pre-cooling, and divided into three groups of 100 fruits each at random with three biological replicates. The first group comprised pepper samples on the day of harvest (fresh; T01 T02 T03), the second group comprised samples stored at 10 °C (control; T04 T05 T06), and the pepper samples stored at 4 °C (chilled samples; T07 T08 T09) were included in the third group. The relative humidity (RH) was 80%. The CI-related physiological parameters were measured at 0, 5, 10, 15, and 18 d. The pepper samples on the day of harvest, and the fruits stored at 4 °C and 10 °C on the 18th day were collected for RNA-Seq. The samples were frozen in liquid nitrogen immediately and stored at −80 °C. All experiments were carried out as soon as the pepper fruits recovered to room temperature.

### 2.2. Measurements of CI index, electrolyte leakage, MDA, and proline

The symptoms of CI include calyx browning and surface pitting. The CI index was estimated on the basis of the percentage of pitting area take up total surface area (Zhao, 2003). Relative electrolyte leakage, which is an important indicator of membrane permeability, was determined according to the method of Mao et al. (2007). The content of MDA, which is the oxidation end-product during lipid peroxidation, was measured according to Meng and Min, (2012). Proline plays a crucial role in tolerance to abiotic stresses. Accordingly, the changes in proline content during storage were investigated as reported by Zhao et al. (2009) with some modification in standing time. The modified shaking time was 30s instead of previous incubation at 23 °C for 24 h.

### 2.3. Total RNA isolation, cDNA library construction, and sequencing

The pepper tissues were sampled from the first group and on the

18th day in the second group and the third group. Total RNA was extracted from pepper tissues using an OmniPlant RNA Kit (CW BIO, Beijing, China), according to the manufacturer's instructions. The integrity and concentration of RNA were determined by 1% agarose gel electrophoresis, OD<sub>260</sub>/OD<sub>280</sub> ratio using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc.) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). High-quality RNA was prepared for the construction of cDNA libraries using the NEBNext® Ultra™ Directional RNA Library Prep Kit (Illumina, USA) according to the manufacturer's protocol. Then, the quality of the cDNA libraries was assessed using the Agilent Bioanalyzer 2100 system. Finally, the cDNA libraries were sequenced using the Illumina HiSeq 4000 platform with paired-end sequencing.

#### 2.4. Mapping reads to the reference genome and gene functional annotation

Clean reads were obtained after discarding the adapter related and low-quality data from raw reads. The genomics of pepper were considered as a reference for sequence alignment and subsequent analysis (<http://peppersequence.genomics.cn/page/species/download.jsp>). The non-redundant unigenes were compared with NCBI non-redundant (NR), Swiss-Prot, Gene Ontology (GO), Cluster of Orthologous Groups (COG), euKaryotic Orthologous Groups (KOG), Protein family (Pfam) and Kyoto Encyclopedia of Genes and Genomes (KEGG) using BLAST. The KEGG Orthology results were obtained with KOBAS2.0. The annotation information of unigenes were obtained by comparing predicted amino acid sequence of unigenes against the Pfam database using HMMER.

#### 2.5. Analysis of differentially expressed genes

The transcript and gene expression level were quantified based on the fragments per kilobase of transcript per million fragments mapped (FPKM); differential expression analysis between samples was performed with DEGseq. During the detection,  $|\log_2(\text{fold change})| \geq 2$  and FDR < 0.01 were set as the threshold to screen significant differentially expressed unigenes.

#### 2.6. Validation of DEGs by RT-qPCR

To verify the reliability of the RNA-Seq data, 20 DEGs were selected to evaluate their gene expression level in bell peppers stored at 4 °C and 10 °C by RT-qPCR. The cDNA was synthesized from total RNA according to the HiFiScript cDNA Synthesis Kit (CW BIO, Beijing, China). The specific primers used for RT-qPCR analysis were designed using Primer 5.0 software; the primer sequences are listed in Table S1. The RT-qPCRs were conducted using UltraSYBR Mixture (CW BIO, Beijing, China) with

a total volume of 20 µl containing 10 µl of 2 × UltraSYBR Mixture, 2 µl of cDNA template, 0.4 µl of primers (0.2 mmol l<sup>-1</sup>) and 7.2 µl of ddH<sub>2</sub>O. The PCR amplification conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min on a QuantStudio™ 5 Real-Time PCR System (ThermoFisher, USA). Three independent biological replicates were carried out for this experiment. The relative transcriptional level was calculated according to the 2<sup>-ΔΔCT</sup> method with a β-actin reference gene as control.

#### 2.7. Statistical analysis

SPSS 20.0 software (SPSS Inc.) was used for statistical analysis and the results for all the experiment are presented as the mean ± standard deviation. Analysis of statistical significance was performed according to one-way analysis of variance (ANOVA) with least significant difference (LSD). Each experiment was repeated three times independently and *P* < 0.05 was considered to represent significance.

### 3. Results

#### 3.1. Changes in phenotype and physiology at low temperatures

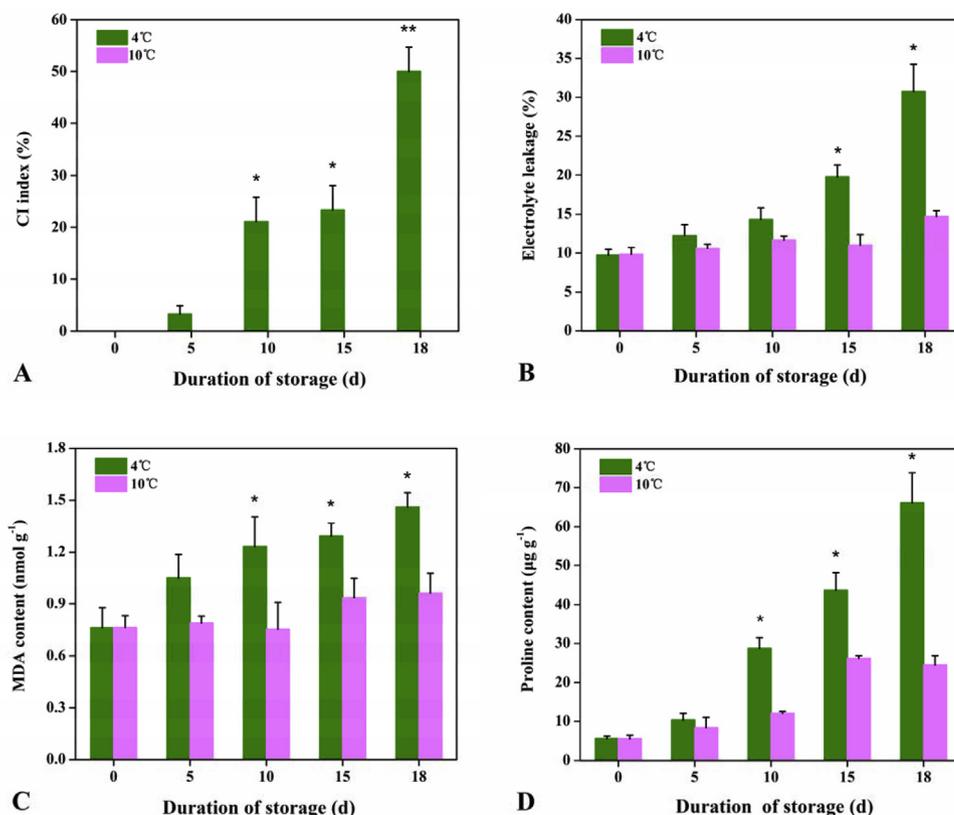
The phenotype of green bell peppers stored at 4 °C and 10 °C were characterized. The control group samples incubated at 10 °C (Fig. 1B) showed no symptoms of chilling injury; however, the bell peppers stored at 4 °C (Fig. 1A) presented typical characteristics of chilling injury such as surface pitting and carpodium brown staining. Further, the CI index of peppers stored at 4 °C significantly increased compared with that in the control (Fig. 2A). To evaluate the physiological damage caused by low temperature, relative electrolyte leakage, proline content, and MDA production were measured. The peppers stored at 4 °C showed a relatively higher electrolyte leakage about 40% during the 18th day (Fig. 2B). This result showed plasma membranes may be damaged under cold stress. Furthermore, proline accumulation in peppers stored at 4 °C was quite significant compared with that in the control (Fig. 2D). On the 18th day, the proline content of samples at 4 °C and 10 °C were 66.2 µg g<sup>-1</sup> and 24.5 µg g<sup>-1</sup>, respectively. In addition to this, the MDA production in the CI group was relatively higher during the later storage period (Fig. 2C). These results indicate that the pepper fruits were more susceptible to cold stress (4 °C) and likely to suffer chilling injury in contrast with the samples stored at 10 °C.

#### 3.2. Sequencing of mRNA and alignment to reference genome

To obtain a comprehensive understanding of the transcriptome connected with gene expression profiles of *C. annuum* fruits during cold stress, nine green pepper fruit samples under distinct storage



Fig. 1. The phenotype of bell peppers on the 18th day of storage at 4 °C (A) and 10 °C (B). Fig. 1A indicate the pericarp pitting and browning of calyxes.



**Fig. 2.** The physiological changes during storage as indicated by CI index (A), electrolyte leakage (B), MDA content (C) and proline content (D) at 10 °C and 4 °C. Each value is the mean of three replicates of 10 fruits and error bars represent the SD of the means (n = 3); \*P < 0.05; \*\*P < 0.01.

temperatures were used for RNA-Seq analysis using the Illumina HiSeq 4000 platform. A full-scale sequencing analysis from nine cDNA samples is shown in Table 1; 61.55 Gb of clean data were obtained, and each library contained 6.19 Gb. Further, the percentages of Q30 base in all samples were greater than or equal to 93.30%. The blast efficiency between our samples and reference genome was relatively high in the range of 84.35%–88.16%, which met the requirements for information analysis.

### 3.3. Comprehensive profiling of transcript expression analysis

To understand the differential expression pattern of unigenes among nine library samples under cold stress in bell peppers, FPKM values were used to calculate gene expression levels via Cufflinks software analysis. On comparing each group, it was found that Set 1 (Fresh vs Control), Set 2 (Fresh vs Chilled), and Set 3 (Control vs Chilled) comprised 1662 (779 up-regulated, 883 down-regulated), 5561 (2606 up-regulated, 2955 down-regulated) and 3863 DEGs (1669 up-regulated, 2194 down-regulated), respectively (Fig. 3A). It was worth noting that

there was a large number of DEGs between the CI group and control, except for Set 2, which indicated that a much larger number of genes was induced under cold stress (4 °C) compared with that in the control (10 °C). In order to better understand the diversity of DEGs between each treatment, a Venn diagram was generated. As is shown in Fig. 3B, there were 199 DEGs in all groups, indicating that the expression level of these genes changed during storage. In addition, 300, 1,920, and 691 DEGs were also identified in Set 1, Set 2, and Set 3, respectively. As can be seen, a large number of genes were induced under cold stress compared with that in the control. Accordingly, Set 3 (Control vs Chilled) was discussed as the main object in the subsequent analysis.

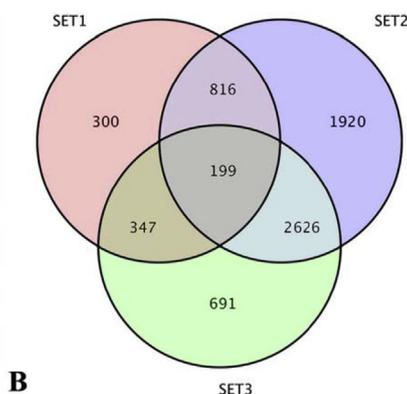
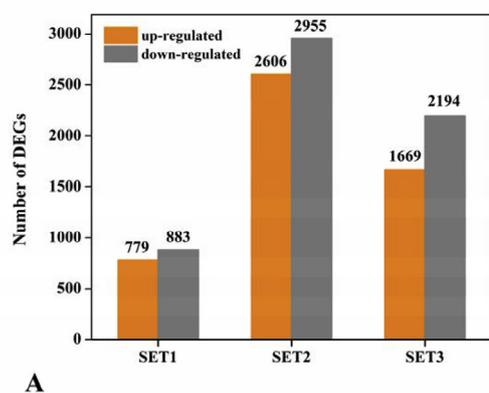
### 3.4. Functional annotation and classification

In order to predict and analyze the function of the unigenes, the identified new genes were searched against the public databases (Swiss-Prot, GO, COG, KOG, Pfam and KEGG) using BLAST software. Of the 4437 unigenes, 3231 unigenes (72.81%) were successfully matched to homologous sequences and corresponding gene annotation information

**Table 1**

Summary of the sequencing data of bell pepper transcriptome.

Sample ID	Clean reads	Clean bases	Q30	GC Content	Mapped Reads	Uniq Mapped Reads
T01	22,157,547	6,623,285,840	94.21%	42.60%	84.35%	81.02%
T02	22,914,278	6,813,756,928	95.58%	44.41%	88.16%	84.73%
T03	22,115,535	6,600,145,722	94.86%	43.08%	87.03%	83.51%
T04	25,045,376	7,498,986,750	93.30%	43.93%	85.56%	75.13%
T05	25,131,886	7,506,459,618	93.97%	43.78%	87.21%	80.65%
T06	23,639,432	7,059,688,788	93.88%	43.13%	87.07%	83.81%
T07	21,747,438	6,497,158,410	94.48%	42.91%	86.50%	82.34%
T08	22,642,414	6,761,003,934	93.85%	42.81%	85.77%	81.41%
T09	20,713,090	6,185,916,814	93.57%	42.58%	85.16%	81.08%



**Fig. 3.** Differentially expressed genes between fresh, chilling injury, and control libraries. (A) The numbers of differentially expressed genes between each two samples. (B) Venn diagram of DEGs between each two samples. SET1 represents Control compared with Fresh, SET2 represents Chilled compared with Fresh and SET3 represents Chilled compared with Control.

was obtained (Table 2). Further, there were 449 (10.12%), 1264 (28.49%), 790 (17.80%), 1616 (36.42%), 1502 (33.85%), 1678 (37.82%), 2900 (65.36%), and 3198 (72.08%) unigenes matched to sequences in the COG, GO, KEGG, KOG, Pfam, Swiss-Prot, eggNOG, and NR databases, respectively. Notably, the match degree of NR databases was the highest: close to 80%.

The GO analysis database is a standard biological annotation system used to classify the functions of *C. annuum* unigenes. Compared with the control (10 °C), a total of 2680 DEGs in the CI group (4 °C) were annotated and classified into 53 functional groups consisting of three categories including biological processes, cell component, and molecular function (Fig. 4A). Among them, the top six GO terms in biological processes were cellular process (2224), metabolic process (2162), single-organism process (2160), response to stimulus (1805), biological regulation (1497), and developmental process (1290). The top six GO terms in cell component were cell part (2209), cell (2209), organelle (1818), membrane (1179), organelle part (781), and membrane part (420). The top six GO terms for molecular function were binding (1607), catalytic activity (1506), transporter activity (230), nucleic acid binding translation factor activity (207), molecular transducer activity (112) and structural molecule activity (30).

In addition, 1398 DEGs (25 clusters) were obtained using the COG database (Fig. 4B). The top three COG terms were general function prediction only (469); replication, recombination, and repair (243); and transcription (242). The lipid transport and metabolism cluster additionally comprised some DEGs.

To further elucidate the functions of the DEGs in response to low temperature systematically, KEGG databases were used to classify and characterize transcriptome DEGs into the corresponding pathway. Compared with the control and CI group, a total of 834 DEGs were enriched in 50 pathways. Among these pathways, the six most significantly enriched were glutathione metabolism, beta-Alanine metabolism, glycosphingolipid biosynthesis - globo series, plant hormone signal transduction, linoleic acid metabolism and steroid biosynthesis in response to cold stress. Moreover, the pathways related to cell membrane lipid metabolism such as glycerolipid metabolism, glycerophospholipid metabolism, and fatty acid metabolism also comprised DEGs involved in low temperature stress.

### 3.5. Identification and analysis of DEGs under cold stress

#### 3.5.1. DEGs involved in lipid metabolism

Phospholipids, as the key component of membrane lipids, play a significant role in maintaining cellular physiological function. Research shows that phospholipid metabolism was strongly induced when plants suffered from temperature-induced stress. Our results showed that 14, 11, and 6 DEGs participated in glycerophospholipid metabolism, glycerolipid metabolism, and sphingolipid metabolism, respectively. Among these DEGs, those that responded to CI included three *PLDs*, one *PLC/DGK*, six *PLAs*, one *DGD*, and one *CERS*, which were significantly

differentially expressed in comparison with the control. Furthermore, there were some related genes such as *LYPLA*, *TGL4* in the above pathways whose expression changed significantly.

Lipid transfer proteins (LTP) are responsible for the transportation of phospholipids between cell membranes and participate in stress resistance under conditions such as low temperature, drought, and salt. Under low temperature stress, 11 DEGs (4 up-regulated, 7 down-regulated) that encode lipid transfer proteins, including nine non-specific LTPs and two phosphatidylinositol/phosphatidylcholine transfer proteins (PI/PC-TP), were identified.

The changes in cell membrane lipid composition are closely related to fatty acid unsaturation in the response to cold stress. In our study, 14 DEGs enriched in fatty acid metabolism were identified; one up-regulated and five down-regulated genes were identified in the fatty acid biosynthesis pathway. Similarly, the fatty acid pathway consisted of three up-regulated and six down-regulated genes. These DEGs mainly encoded FabF, FabG, FATB, Des, FadD and MFP2 proteins.

#### 3.5.2. DEGs related to dehydration and osmoregulation

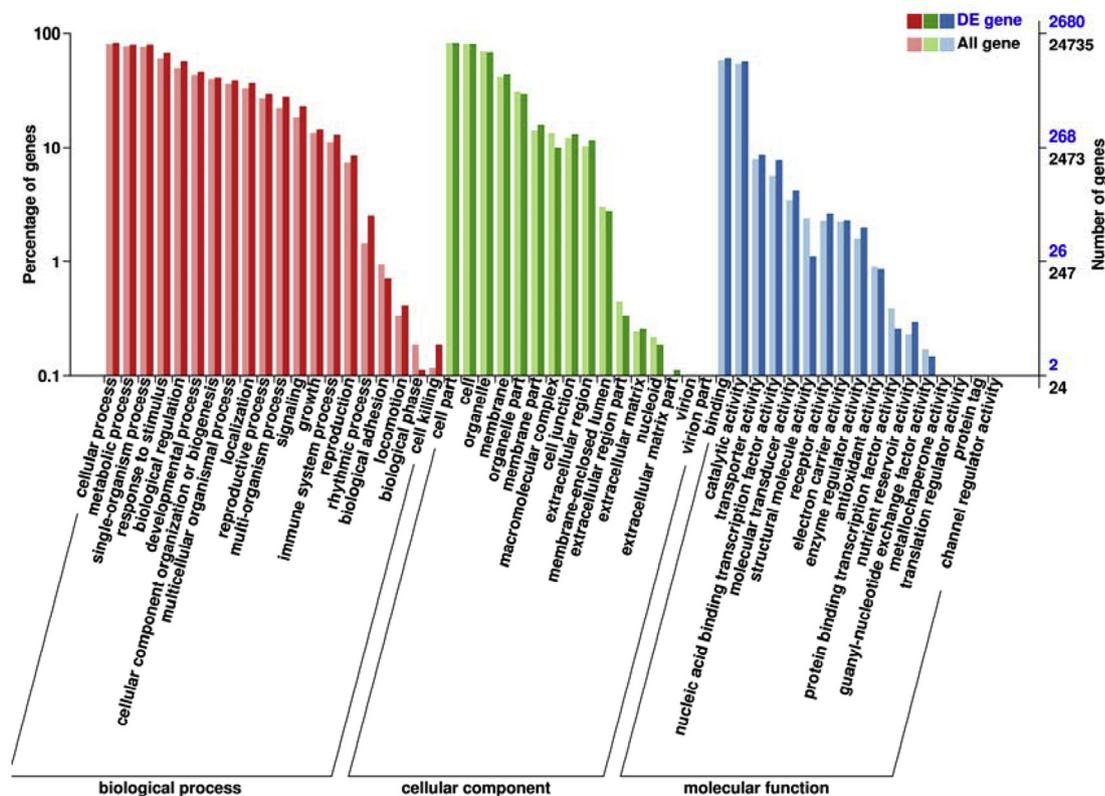
In general, plants are also indirectly subject to dehydration stress caused by low temperature. Our previous results showed that the moisture content in CI group samples declined significantly (Kong et al., 2018). Similarly, analysis of differentially expressed genes indicated that five genes that encoded aquaporins and four genes that encoded dehydration-responsive proteins were changed distinctly. All five aquaporin-encoding genes were down-regulated and one dehydration-responsive protein encoding gene was up-regulated.

Dehydration stress is often accompanied by osmotic adjustment. Plants need to accumulate organic or inorganic substances such as glycine betaine and proline to maintain their capacity to hold cellular moisture. According to the pathway enrichment analysis, 'arginine and proline metabolism', which is activated by cold-induced dehydration, results in a high number of DEGs. Fourteen (5 up-regulated, 9 down-regulated) DEGs were enriched in this pathway. In addition, the genes that encode proline-rich protein were also significantly differentially expressed.

**Table 2**

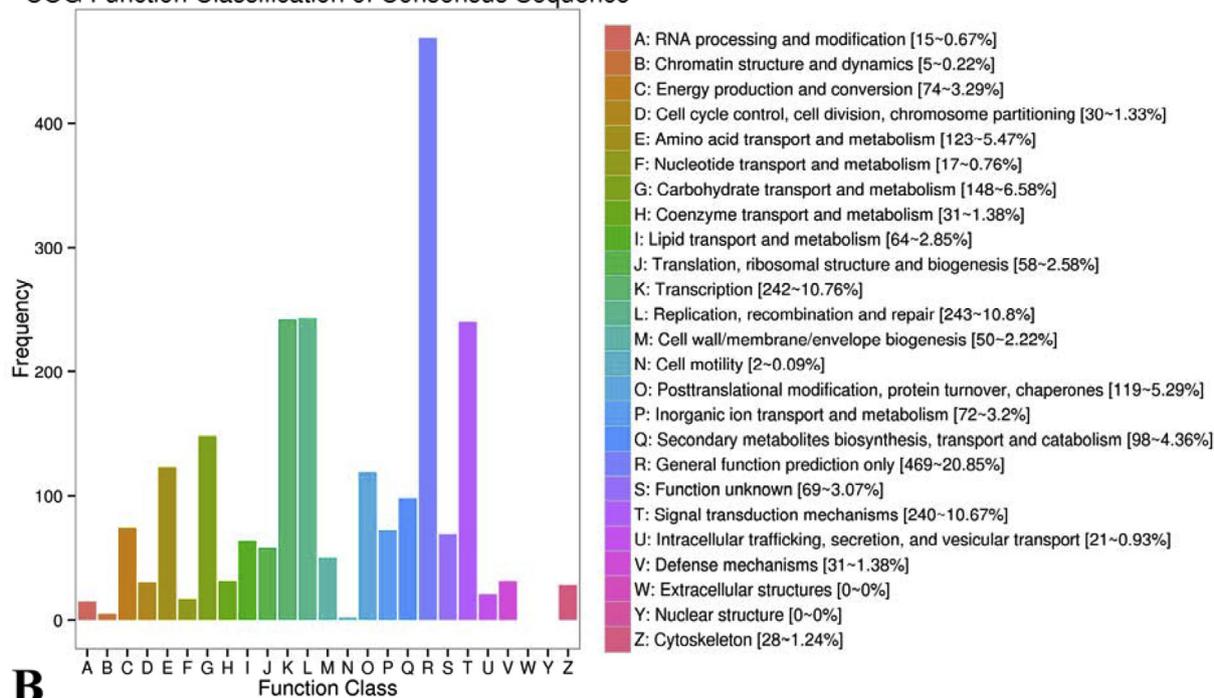
Overview of annotated DEGs for the bell pepper transcriptome.

Database	Total unigenes	Annotated unigenes	Percent (%)
COG	4437	449	10.12%
GO	4437	1264	28.49%
KEGG	4437	790	17.80%
KOG	4437	1616	36.42%
Pfam	4437	1502	33.85%
Swiss-Prot	4437	1678	37.82%
eggNOG	4437	2900	65.36%
NR	4437	3198	72.08%
All annotated unigenes	4437	3231	72.81%



**A**

**COG Function Classification of Consensus Sequence**



**B**

Fig. 4. Functional annotations and classifications of unigenes in the CI group (4 °C) and control (10 °C) in GO (A) and COG (B) databases.

**3.5.3. DEGs related to plant hormone signal transduction pathway**

KEGG enrichment analysis results showed that there were 50 DEGs (10 up-regulated and 40 down-regulated) involved in plant hormone signal transduction pathway under chilling stress in bell pepper fruits.

The signal transduction pathways, including auxin, cytokinin (CK), gibberellin (GA), abscisic acid (ABA), ethylene (ET), brassinosteroid (BR), jasmonic acid (JA) and salicylic acid (SA), comprised 17, 4, 5, 10, 5, 1, 5 and 3 DEGs respectively. These results indicate that multiple

processes in plant hormone signal transduction pathways are activated under cold stress in bell peppers. The details of the primary DEGs above are listed in Tables 3–5.

### 3.6. Changes in the expression of transcription factors in response to cold stress

Transcriptional regulation is an important mechanism in eukaryotic gene expression that plays an essential role in abiotic stress. The transcriptome data indicated that, compared with that in the control, a total of 250 TFs, including 90 up-regulated and 160 down-regulated TFs, were differentially expressed under cold stress. These TFs were divided into 41 different families, which mainly included AP2/ERF, MYB, NAC, C2H2, WRKY, HB-HD-ZIP, and bHLH (Fig. 5). Among them, up-regulated TFs families mostly comprised NAC (14%), C2H2 (12%) and AP2/ERF (12%). Besides, the GO annotation information of up-regulated MYB, NAC, bHLH, WRKY and AP2/ERF members in biological process were presented in Table S2 as supplement. Among of them, most TFs participated in response to cold, water deprivation and abscisic acid-activated signaling pathway.

### 3.7. Validation of the RNA-Seq results by RT-qPCR

To further confirm gene expression patterns of each differentially expressed transcript obtained by RNA-Seq, we manually selected 20 unigenes with annotations for RT-qPCR determination (Fig. 6). These candidate genes were closely associated with membrane lipid metabolism and fatty acid metabolism. Results showed that the expression profiles of the 20 genes, obtained via RT-qPCR, had a high similarity with the RNA-Seq data. These findings confirm the reliability of the differential expression analysis data obtained by RNA-Seq, and reflect

**Table 3**

Gene related to lipid metabolism in response to low temperature.

Gene ID	log2FC	regulated	Gene description
Capana00g003956	-3.57	down	Phospholipase D
Capana03g000192	3.73	up	Phospholipase D epsilon
Capana01g000355	2.11	up	Phospholipase PLDβ1
Capana08g000231	4.50	up	Phospholipase A1-Igamma1
Capana02g003128	2.53	up	Patatin-like phospholipase 1
Capana01g004371	3.65	up	Phospholipase A1-Ibeta2
Capana01g000686	5.39	up	Phospholipase A1-Ibeta2
Capana06g001615	-6.99	down	Phospholipase A1-Ibeta2
Capana02g001805	-3.82	down	Phospholipase A1-IIgamma-like
Capana12g002911	2.24	up	Diacylglycerol kinase 2
Capana08g000861	2.23	up	Digalactosyldiacylglycerol synthase 2
Capana03g000932	4.52	up	Protein ASC1
Capana01g000777	-2.00	down	PRP1 precursor
Capana02g001862	-7.89	down	Non-specific lipid-transfer protein 1
Capana06g000094	-2.58	down	Non-specific lipid-transfer protein-like protein
Capana01g003235	-6.41	down	Non-specific lipid-transfer protein-like protein
Capana11g000523	2.40	up	Non-specific lipid-transfer protein-like protein
Capana08g001783	4.45	up	Leucine-rich repeat extensin-like protein 5
Capana01g003236	-6.47	down	Non-specific lipid-transfer protein-like protein
Capana08g002006	-2.39	down	Non-specific lipid-transfer protein-like protein
Capana10g001554	3.41	up	Non-specific lipid transfer protein precursor
Capana08g000093	-5.46	down	SEC14 cytosolic factor
Capana12g002893	2.39	up	Phosphatidylinositol/phosphatidylcholine transfer protein SFH1-like
Capana06g001001	6.14	up	Short-chain type dehydrogenase/reductase-like
Capana12g002131	3.83	up	Very-long-chain enoyl-CoA reductase-like
Capana03g002638	-2.39	down	3-oxoacyl-[acyl-carrier-protein] synthase II
Capana03g001691	-2.30	down	Palmitoyl-acyl carrier protein thioesterase
Capana12g002555	-4.97	down	Stearyl-acyl carrier protein desaturase
Capana00g003654	-3.53	down	Long chain acyl-CoA synthetase 8-like
Capana01g002806	-2.34	down	Long chain acyl-CoA synthetase 1-like
Capana08g000853	2.04	up	Peroxisomal fatty acid beta-oxidation multifunctional protein AIM1-like
Capana00g003264	-5.93	down	Glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a-like
Capana12g000150	-3.38	down	Glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a-like
Capana05g000220	-2.43	down	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase PASTICCINO 2-like
CapanaNewGene14567	-4.67	down	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase PASTICCINO 2A-like

**Table 4**

Genes related to dehydration and osmoregulation in response to low temperature.

Gene ID	log2FC	regulated	Gene description
Capana12g001128	-3.32	down	Aquaporin PIP-type pTOM75-like
Capana09g002162	-2.80	down	Aquaporin PIP2-1-like
Capana06g001982	-3.97	down	Aquaporin PIP2-7-like
Capana06g000557	-3.58	down	Aquaporin-like protein
Capana11g000752	-2.87	down	Aquaporin TIP1-3
Capana09g001688	-4.32	down	Dehydration-responsive protein RD22
Capana00g004634	4.04	up	Dehydration-responsive element-binding protein 2A-like
Capana06g000924	-2.91	down	Protein DEHYDRATION-INDUCED 19 homolog 5-like
Capana03g000268	-3.25	down	Dehydration-responsive element-binding protein 3-like
Capana06g001007	-2.93	down	Delta-1-pyrroline-5-carboxylate dehydrogenase 12A1
Capana01g000777	-2.00	down	PRP1 precursor
Capana12g002880	-3.66	down	Proline-rich protein
Capana08g002089	3.97	up	Proline-rich protein
Capana12g002881	-3.23	down	Proline-rich protein

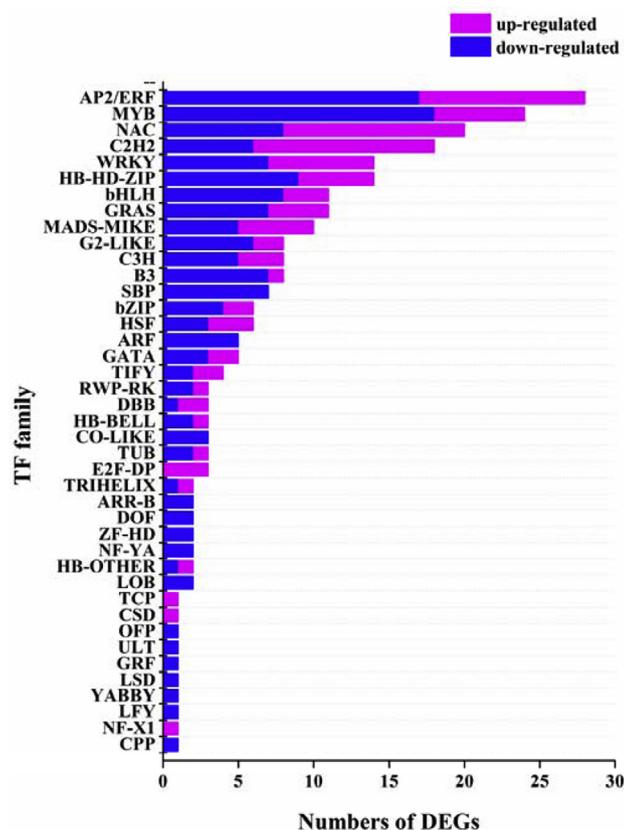
the actual transcriptomic changes in bell peppers under cold stress.

## 4. Discussion

Subtropical plants are more vulnerable to CI during storage and transportation. As a result, pepper fruits exhibit symptoms such as pitting, seed browning, and even decay. This CI phenomenon may be caused by cell metabolic disorders during cold stress. To better understand the mechanism of chilling injury in *C. annuum* L. at the molecular level, comprehensive transcriptome profiling was carried out at 4 °C

**Table 5**  
Gene related to plant hormone signal transduction in response to low temperature.

Gene ID	log2FC	regulated	Gene description
Capana05g002062	-4.61	down	Abscisic acid receptor PYL4-like
Capana06g001547	-7.06	down	Abscisic acid receptor PYR1-like
Capana12g001078	-2.03	down	Abscisic acid receptor PYR1-like
Capana00g003839	-2.01	down	Probable protein phosphatase 2C 51-like
Capana05g002193	-2.26	down	Protein phosphatase 2C 37-like
Capana06g000398	-4.31	down	Protein phosphatase 2C
Capana05g000287	-2.10	down	Serine/threonine-protein kinase SAPK2-like
Capana00g004754	2.06	up	Abscisic acid-insensitive5-like protein 2
Capana08g002366	-4.12	down	Abscisic acid-insensitive 5-like protein
Capana08g001036	-3.07	down	Probable indole-3-acetic acid-amido synthetase GH3.5
Capana10g000854	-3.62	down	Probable indole-3-acetic acid-amido synthetase
Capana03g000117	2.13	up	Protein TIFY 10B-like
Capana09g000144	2.97	up	Protein TIFY 10B-like
Capana01g001914	-2.04	down	Protein TIFY



**Fig. 5.** The numbers of differentially expressed TFs responsive to cold stress (4 °C) compared with that in the control.

and 10 °C using the RNA-Seq technique. A large number of DEGs involved in diverse cell, molecular and biological pathways were identified during the CI period. These transcriptome analysis results provide key points to explain the mechanism underlying the metabolic changes in bell peppers at the molecular level in response to cold stress.

#### 4.1. Cold-responsive genes associated with the biomembrane system

When the environmental temperature decreases, a physical phase change occurs in the cell membrane, which gradually changes from

liquid crystalline phase to solid crystalline state. This is usually accompanied by the increase in membrane permeability, which is mainly reflected in changes in electrolyte leakage. In this study, the higher electrolyte leakage in the CI group revealed that the cytomembrane has been damaged due to cold stress. Membrane lipid composition and content alters to adapt to environment temperature; however, when the self-defense mechanism cannot defend against low temperature stress, membrane lipids begin to degrade. Phospholipases, including PLC, PLD, PLA<sub>1</sub> and PLA<sub>2</sub>, hydrolyze phospholipids into various products including phosphatidic acid (PA), diacylglycerol (DAG), free fatty acids (FFAs), and lysophospholipids (LPLs), which play a crucial part in membrane lipid metabolism (Hong et al., 2016). In our study, we found that three *PLD* genes, five *PLA*<sub>1</sub> genes, and one *DGK* gene were differentially expressed when chilling injury occurred. These findings indicate the activation of phospholipase-encoding genes in response to cold. Similar results for the expression of the phospholipase gene family under cold stress have also been demonstrated in plants such as *Arabidopsis thaliana* and the tea plant (Tasma et al., 2008; Li et al., 2018). Moreover, the genes that encode digalactosyldiacylglycerol synthase (DGD) and alpha-galactosidase (galA) are differentially expressed in glycerolipid metabolism. Further, we found that the ceramide synthetase (CERS)-encoding gene, which acts as a sphingosine hydrolase during sphingolipid metabolism, was up-regulated.

In addition to the changes in membrane permeability, membrane fluidity is altered when plants are subjected to low temperature stress. Generally, membrane fluidity declines as the temperature decreases. The underlying mechanism involves the alteration of the fatty acid composition of membrane lipids via the transition of carbon atoms or carbon-carbon double bond numbers to increase fatty acid unsaturation. Our study showed that the fatty acid metabolism pathway was triggered, and numerous related genes were differentially expressed. In the unsaturated fatty acid synthesis pathway, the genes encoding *fabG* and *CER10* were up-regulated; in contrast, the genes encoding *PAS2*, *desA1*, and *desC* were down-regulated. Notably, the significantly down-regulated desaturase synthetic genes (*desA1* and *desC*) indicated that chilling injury disrupted the internal desaturation mechanism, resulting in the cell membrane becoming less fluid. The changes in the expression of these genes during fatty acid metabolism have additionally been demonstrated in other plants such as ‘Nanguo’ pear and *Nostoc* (Shi et al., 2018). Furthermore, another cause of the decreasing fluidity is the modification of membrane protein properties due to the combination of accumulated MDA and protein caused by membrane lipid peroxidation. Similarly, our physiological results showed a higher MDA content in the CI group.

LTP is a micromolecule secretory protein, which is abundant in plants. These proteins can bind lipids such as fatty acids, phospholipid, glycolipid, sterol, to alter the membrane lipid composition. Recent studies show that LTP plays an important role in plant adversity stress. Hairat et al. (2018) found that TaLTP40- and TaLTP75-overexpressing transgenic *Arabidopsis* showed a constitutively enhanced salt tolerance. Their research also indicates that TaLTP expression may be driven by changes in membrane fluidity, and could be involved in transferring membrane lipids to the biological membranes, thus imparting tolerance to various abiotic stresses. Further, Lin et al. (2013) demonstrated that LTP3 acts as a target of MYB96 and is involved in plant tolerance to freezing and drought stress. In our research, 9 DEGs that encoded LTP and 2 DEGs encoded PI/PC-TP were identified. Four of these genes were up-regulated significantly, which revealed that LTP was involved in lipid metabolism regulation in response to cold stress.

#### 4.2. Cold-responsive genes related to dehydration and osmoregulation

Most plants exhibit symptoms of dehydration in response to chilling stress; this is known as dehydration stress. This occurs as a result of cell membrane damage and results in an increase in membrane permeability. Studies have shown that some plants, such as cucumbers and

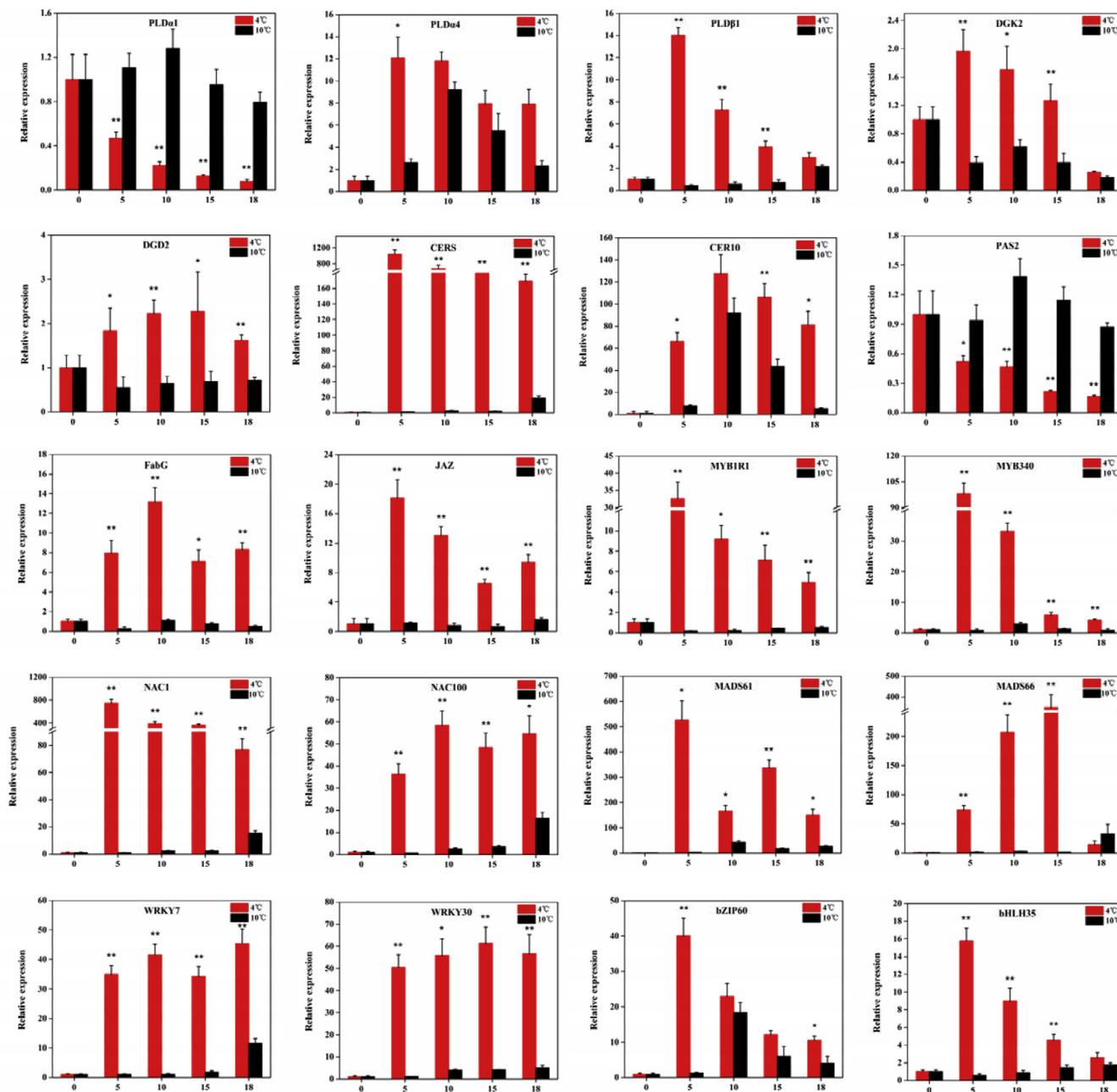


Fig. 6. Validation of the RNA-Seq results by RT-qPCR of twenty selected genes during storage. The relative transcription level was calculated according to the  $2^{-\Delta\Delta CT}$  method with a  $\beta$ -actin reference gene as control. Each value is the mean  $\pm$  SD of three replicates; \* $P < 0.05$ ; \*\* $P < 0.01$ .

zuchinis, show obvious surface pitting and dehydration during cold stress; similar results were observed in bell peppers. Dehydration-responsive protein and aquaporins are crucial for this metabolic process. Aquaporins are mainly responsible for transmembrane water transportation, and many studies show that aquaporins participate in abiotic stress (Ahamed et al., 2012). For instance, transgenic banana plants overexpressing *MusaPIP1;2* displayed enhanced tolerance to both cold and drought stress (Shekhawat and Ganapathi, 2013). In our research, 3 DEGs that encoded PIPs, and 2 encoding TIPs were completely down-regulated in the CI group. Besides the aquaporins, dehydration-responsive protein also plays an important role in response to cold stress. To date, studies suggests that the accumulation of dehydration-responsive protein is closely related to resistance to dehydration in plants cells. In our study, 2 genes encoding dehydration-responsive element-binding protein, 1 encoding dehydration-responsive protein RD22, and 1 encoding dehydration-induced protein 19 were differently

expressed, whereas only the dehydration-responsive element-binding protein 2A coding gene was up-regulated. This result explains the declining moisture content and increased electrolyte leakage in CI group fruits. Thus it can be seen that the dehydration-responsive protein coding gene is involved in cold stress response. These findings are consistent with the data for the gene expression of *ZmDREB2A* under cold stress in maize (Nguyen et al., 2009).

Proline plays a crucial osmoprotective role against chilling injury in plants. The synthesis of proline increases notably to maintain water potential in the cell when plants are subject to abiotic stress such as salt stress, osmotic stress, and cold stress (Liang et al., 2013). Our research showed an apparent increase in proline content at 4 °C compared with the control. Accordingly, differential expression analysis of genes related to proline metabolism was performed. We found that genes encoding aldehyde dehydrogenase (ALDH), polyamine oxidase (PAO), ornithine decarboxylase, prollyl 4-hydroxylase (P4HA) and arginine

decarboxylase were up-regulated, and that encoding 1-pyrroline-5-carboxylate dehydrogenase (P5CDH) was down-regulated. P5CDH, which is the key enzyme in the proline degradation pathway, catalyzes the degradation of P5C to generate Glu. Reduction in proline degradation is a major cause of proline accumulation. Furthermore, four proline-rich protein-coding genes in our study were also differentially expressed, which was the same as that observed in cotton and oilseed rape during cold stress (Huang et al., 2011; Priyanka et al., 2010). Therefore, we can infer that cold stress in bell peppers induces the expression of genes associated with dehydration and osmoregulation.

#### 4.3. Cold-responsive genes involved in plant hormone signal transduction

Although plants contain infinitesimal quantities of hormones, they play a crucial role in the anti-stress physiology of plants. In our study, the plant hormone signal transduction pathway comprised the maximum amount of DEGs under chilling stress. Among these, auxin and ABA were the two primary transduction pathways owing to their highly enriched DEGs. There were 4 up-regulated DEGs and 13 down-regulated DEGs in the auxin signal transduction pathway; 2 up-regulated DEGs and 8 down-regulated were enriched in ABA signal transduction pathway. Five DEGs were assigned to the GA, ET, and JA pathways.

Research shows that ABA promotes the transportation of water, increases the content of osmoregulation substances, and closes stomata rapidly to reduce water loss (Lata and Prasad, 2011). A previous study shows that PA, the biomarker of chilling injury, participates in ABA-mediated pathways (Zhang et al., 2004). PA binds ABI1 to the plasma membrane and inhibits its translocation to the nucleus, where ABI1 interacts with the transcription factor ATHB6, which is a negative regulator of the ABA response (Hou et al., 2016). Consistent with these data, genes that encoded phosphatase 2C (PP2C) and ABA-responsive element binding factor (ABF) were differentially expressed in this study. In addition, the JA-mediated signal transduction pathway plays an important role in multiple stress responses. A previous report demonstrated that jasmonate regulates the ICE-CBF/DREB1 transcriptional pathway as a critical upstream signal to avoid freezing injury in *Arabidopsis* (Hu et al., 2013). In our research, one gene encoding jasmonic acid-amino synthetase (JAR1) and three genes that encode jasmonate ZIM domain-containing protein (JAZ) were also differentially expressed. The above results indicate that the plant hormone signal transduction pathway is greatly induced under cold stress in bell peppers.

#### 4.4. TFs responsive to low temperature

Transcription factors play a central role in stress signaling pathways in plants, during which they may be directly involved in the regulation of downstream target gene expression in response to the external environment. To date, more than 1500 TFs have been identified including NAC, bZIP, MYB, WRKY, and AP2/ERF in the *Arabidopsis* genome (Riechmann et al., 2000). Some of these TFs have been confirmed as crucial regulators of the response to cold stress (Zhao et al., 2016). In this study, 250 DEGs that encoded TFs encompassing 41 families were identified and classified during chilling of pepper fruits. Among these genes, AP2/ERF, MYB and NAC families were mainly involved in the response to low temperature. These results had little comparability with the findings in hot pepper by Hwang et al. (2005). Furthermore, specific TFs such as C2H2, bHLH, GRAS, and HB-HD-ZIP were also identified in our research. Numerous studies have showed that AP2/ERF, MYB, and NAC TFs have significant effect on plant growth and abiotic stress such as drought stress, salt stress, hypoxia stress and cold stress (Seo et al., 2009). A recent study has reported that *OsPLDα1* is directly regulated by OsDREB1A specific to cold responses in rice, thus likely providing positive feedback regulation of the cold signal transduction pathway (Huo et al., 2016). A study in banana also found that MYBS3

could repress the well-known ICE1-CBF-dependent cold signaling pathway, and that MaCBF1 and MaCBF2 genes are repressed at the transcriptional level after cold treatment (Dou et al., 2016). Our current research further confirmed that AP2/ERF, MYB, and NAC TFs play an important role in the response to low temperature, and regulate the expression of downstream genes in bell peppers.

In addition to the above TFs families, other TFs families such as WRKY, HB-HD-ZIP, bHLH, bZIP, HSF, and ARF were also identified in bell peppers, suggesting that a large number of TFs were induced as transcriptional regulators to activate the expression of downstream target genes in response to cold stress. Further studies are needed to elucidate the functions and target gene-regulatory mechanisms of these TFs in response to cold stress.

## 5. Conclusion

In conclusion, in the present study, a comprehensive transcriptome profile of bell peppers under cold stress was obtained using RNA-Seq technology. Phenotypic and physiological changes during the storage period were determined and biomembrane damage was verified. Additionally, numerous potential genes and TFs related to the biomembrane system, dehydration and osmoregulation, and plant hormone signal transduction processes were identified. These DEGs may play a crucial role in the response to low temperature in bell peppers. Further, the study provides novel insights into a series of molecular mechanisms underlying physiological metabolism and defense. In future work, we aim to focus on the molecular regulatory mechanisms specific to structural genes and TFs associated with membrane lipid metabolism.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.03.033>.

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## Conflicts of interest

None.

## Authors' contributions

Shu-juan Ji, Xi-man Kong, Qian Zhou and Bao-dong Wei designed the experiment. Xi-man Kong performed the experiment and data analysis. Feng Luo, Ya-juan Wang, Hua-jun Sun and Ying-bo Zhao contributed in providing chemicals, reagent, analyses, and tools. All authors read and approved the final manuscript.

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