Identification of common and specific cold resistance pathways from cold tolerant and non-cold tolerant mango varieties (#101295)

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Identification of common and specific cold resistance pathways from cold tolerant and non-cold tolerant mango varieties

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In recent years, mango has frequently encountered severe climate and environmental challenges such as low temperatures, seriously affecting the sustainable development of the industry. In order to reveal the possible molecular mechanism of cold resistance, physiological measurements showed that the activities of superoxide dismutase (SOD) and peroxidase (POD) were found to be higher in Jinhuang (JH) mango plants than that of Tainong (TN) mango plants under cold stress, indicating the cold tolerant (JH) and non-cold tolerant (TN) mango varieties were firstly determined for further evaluation. Subsequently, transcriptomics showed 8,337 and 7,996 differentially expressed genes (DEGs) were respectively identified in JH and TN mango varieties treated at 4℃ for 36 hrs, while more DEGs (10,683 and 10,723) were screened when treated at low temperature for longer. Quantitative real-time PCR (qRT-PCR) of the selected DEGs confirmed their transcriptional levels displayed agreement to the transcriptome data. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed two primary cold resistant regulation pathways, photosynthesis-antenna proteins pathway and photosynthesis pathway, were both significant annotated in the two mango varieties, indicating share the common regulation mechanism response to cold stress. Furthermore, five specific cold resistant pathways, such as amino acid and carbohydrate metabolisms, were identified in JH mango variety, indicating the specific regulation pathways in cold tolerant mango varieties. These results provided insights into the primary and specific molecular mechanisms of different mango variety resistance to chill.

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Abstract

In recent years, mango has frequently encountered severe climate and environmental challenges such as low temperatures, seriously affecting the sustainable development of the industry. In order to reveal the possible molecular mechanism of cold resistance, physiological measurements showed that the activities of superoxide dismutase (SOD) and peroxidase (POD) were found to be higher in Jinhuang (JH) mango plants than that of Tainong (TN) mango plants under cold stress, indicating the cold tolerant (JH) and non-cold tolerant (TN) mango varieties were firstly determined for further evaluation. Subsequently, transcriptomics showed 8,337 and 7,996 differentially expressed genes (DEGs) were respectively identified in JH and TN mango varieties treated at 4℃ for 36 hrs, while more DEGs (10,683 and 10,723) were screened when treated at 4℃ for 72 hrs. Quantitative real-time PCR (qRT-PCR) of the selected DEGs confirmed their transcriptional levels displayed agreement to the transcriptome data. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed two primary cold resistant regulation pathways, photosynthesis-antenna proteins pathway and photosynthesis pathway, were both significant annotated in the two mango varieties, indicating share the common regulation mechanism response to cold stress. Furthermore, five specific cold resistant pathways, such as amino acid and carbohydrate metabolisms, were identified in JH mango variety, indicating the specific regulation pathways in cold tolerant mango varieties. These results provided insights into the primary and specific molecular mechanisms of different mango variety resistance to chill.

Keywords: Mango, abiotic stress, cold resistance, omics.

1. Introduction

Mango (*Mangifera indica* L.) is a tropical fruit with rich nutritional value, such as vitamin C, carotene, dietary fiber, etc. Its pulp is delicate and juicy, and releases a unique aroma and sweetness, so it is loved by people around the world (Lebaka et al., 2021). China 's mango industry has experienced decades of development and has now become one of the world 's major mango producing countries. It is widely planted in tropical and subtropical regions of China, including Hainan, Guangdong, Guangxi, Yunnan and Sichuan.

In nature, plants often suffer from biotic stress of diseases, pests and weeds, and abiotic stress such as high temperature, low temperature, drought and salinity, which seriously affect the yield and quality of crops (Dofuor et al., 2023; Mulungu et al., 2023; Kan et al., 2023; Islam et al., 2022). Temperature is the main decisive factor affecting plant growth and regional distribution of plants (Zhang et al., 2023), and it is of great significance to study its impact on tropical agricultural production and food security. Mango is a tropical evergreen fruit tree, which prefers high temperature. The optimum growth temperature for mango is among 24~27℃. The plants and their fruits will be damaged by cold stress when the temperature is lower than 4℃, especially young trees with high sensitive to the low temperature (Liu et al., 2023). Furthermore, the mango planting area in China belongs to the northern margin of the earth. In winter, it is periodically threatened by natural disasters such as cold damage or frost, which has become an important factor affecting mango production in China (Kong et al., 2024). In recent years, mango has frequently encountered severe climatic and environmental challenges, which causes huge economic losses to farmers or agricultural enterprises and seriously affects the sustainable development of the industry.

The damage caused by low temperature to plants can be divided into cold damage and freezing damage. Cold damage affects plant photosynthesis, inhibits intracellular enzyme activity, imbalances between reactive oxygen species and endogenous hormones, and even causes cell death in severe cases. Freezing damage can cause freezing inside the cell, and ice crystals can puncture the cell membrane, resulting in cell dehydration, cell death and tissue necrosis, and even plant death (Hincha and Zuther, 2020; Takahashi et al., 2018). Mango fruit trees are perennial plants. Therefore, severe cold and freezing damage will not only cause death of flower buds, leaves and branches in the year, but also weaken the tree, leading to leaves rot, dry rot, etc., affecting the growth of the following years. Studies have been shown that the damage of low temperature to fruit trees mainly destroys the structure of cell membrane, thus reducing the fluidity of membrane and leading to the inactivation of ATPase in plasma membrane. At the same time, it also causes membrane lipid phase transition, changes the arrangement of membrane proteins and membrane lipids, and increases the permeability of the cells. Then, the cells are subjected to dehydrated, osmosis and mechanically damaged. Eventually, the physiological and

92 biochemical processes of the plant cell membrane structure are continuously disrupted, resulting
93 in plant death (Kidokoro et al., 2022).

94 However, plants can adapt to low temperature and cold environment through genetic variation
95 and natural selection for a long time, thus evolving cold resistance pathways (Sasaki and Imai,
96 2023). At present, high-throughput omics combined analysis methods such as genomics,
97 transcriptomics, proteomics and metabolomics have been widely used to study different abiotic
98 stresses in plants and deepen the understanding of the different biological regulation pathways.
99 Transcriptome high-throughput sequencing, as a modern genetic research tool, is widely used in
100 animals and plants to analyze the mRNA expression of organisms in specific biological
101 processes and reveal their internal molecular mechanisms. So far, many studies have been
102 carried out on the transcriptional response of plants to low temperature stress, including
103 pineapple (Hong et al., 2023), banana (Zhu et al., 2023) and other tropical crops. These studies
104 have found a large number of cold stress-related genes involved in adapting to low temperature
105 changes. Some scholars have reported that molecular process responses to low temperature stress
106 in many plants, including gene expression , redox state and complex signal transduction (Gan et
107 al., 2019).

108 At present, the research on cold resistance of mango mainly focuses on the identification and
109 evaluation of cold resistant germplasm resources, cold resistance evaluation methods,
110 physiological and biochemical determination, cold resistance gene mining and cold prevention
111 and control measures (Sudheeran et al., 2018; Yamanaka et al., 2019; Gafni et al., 2022; Zhang
112 et al., 2023). However, the research progress on the molecular mechanism of cold resistance of
113 mango is relatively slow. In this study, to provide a theoretical basis for the genetic improvement
114 of mango cold resistance traits and the prevention of cold and freezing in production, the non-
115 cold resistant Tainong mango and the cold-resistant Jinhuang mango varieties were used as
116 experimental objects, and the genes response to low temperature stress and the cold-resistant
117 KEGG pathway were analyzed to determine the cold-resistant regulation mechanism by
118 comparing transcriptomics and molecular biology methods. This study provides new insights
119 into the adaptation of mango to low temperature to promote the breeding of tropical plants stress-
120 resistant varieties.

121

122 ## 2. 2. Materials and Methods

123 ### 2.1 Plant materials and Cold stress

124 Two mango varieties, Jinhuang (cold tolerant; JH) and Tainong (cold-sensitive; TN), were used
125 to analyze the defense mechanisms against cold stress. The 30 seedlings of JH (61.20 cm ±8.35)
126 and TN (57.00 cm ±6.75) plants were obtained from Hainan Baizhou Agriculture Co., LTD,

Danzhou, China. These seedlings were being added 50 mL of H_2O and were pre-treatment in a greenhouse at 24℃ for 7 days. The 15 seedlings of JH were devided into three groups on average, namely JH4-1 group, JH4-2 group and JH24 group, while the 15 seedlings of TN were devided into three groups on average as well, namely TN4-1 group, TN4-2 group and TN24 group.

Among these groups, JH4-1, JH4-2, TN4-1, and TN4-2 were placed in a low temperature culture room at 4℃ for 36 hrs (JH4-1 and TN4-1 groups) or 72hrs (JH4-2 and TN4-2 groups) for cold stress treatment. The two control groups of JH24 and TN24 were maintained in the greenhouse at 24℃ for 72 hrs. Treated samples were collected at 36 hrs (JH4-1 and TN4-1 groups) or 72 hrs (JH4-2 and TN4-2 groups). Control samples were also collected from the JH24 group and TN24 group. Five leaf samples were collected from each group. All the samples were immediately frozen in liquid nitrogen and stored at −80 ℃ for further use.

2.2 Superoxide dismutase (SOD) and Peroxidase (POD)

In order to determine the changes of SOD and POD activities of JH mango and TN mango varieties under adverse conditions. Each leaf of 30 mango plants was selected after cold stress, and every three leaves from the same group were mixed for evaluating some physiological indices in both mango varieties. The activities of SOD (Catalog No. G0101F, Grace, Suzhou, China) and POD (Catalog No. G0107F, Grace, Suzhou, China) were measured using the kits provided by Suzhou Grace Biotechnology Co., Ltd.

2.3 RNA preparation and qualification

Total RNAs of mango leaves were extracted by using RNAprep Pure Plant Plus Kit (Polysaccharides & Polyphenolics-rich) according to the manufacturer's instruction (Catalog No. DP441, TIANGEN, Beijing, China), and further treated with DNase I (Catalog No. RT411, TIANGEN, Beijing, China) to remove DNA contamination. The RNA concentration and purity were measured using Nanodrop2000 (ThermoFisher Scientific, MA, USA). The RNA integrity (RIN) was assessed using the Caliper LabChip GX system (PerkinElmer, MA, USA).

2.4 Library preparation for transcriptome sequencing

A total amount of ~ 3.5 μg RNA per sample was used as input material for the cDNA library construction. Sequencing libraries were generated using Hieff NGS Ultima Dual-mode mRNA Library Prep Kit (Catalog No. 13533ES96, YEASEN, Shanghai, China) as following three steps: Firstly, the enrichment, purification, and fragmentation of mRNA were performed. Then, the first strand cDNA was synthesized for preparing the second strand cDNA, which includes end repair and dA-tailing. Thirdly, adaptor primers adpter3 and adpter5 (Table 1) were linked to the two ends of ds cDNA and the products were purified. Finally, the cDNA libraries were further

amplified and purified for the library quality inspection by using Qsep400 standard DNA clip kit in Qsep-400 instrument.

The prepared libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, USA) using NovaSeq 6000 S4 Reagent Kit (Illumina, San Diego, USA), and 150-bp paired-end reads were generated.

2.5 Data quality control of the transcriptomic data

Based on sequencing-by-synthesis (SBS) technology, the significant amounts of raw data with high-quality were generated. Raw data was saved in fastq format. Raw reads in the fastq format were firstly cleaned to remove the adaptor sequences and low quality reads (ploy-Ns ratio greater than 10%, or bp value of Q≤10 greater than 50%), generating the clean data. Q30 and GC content of the clean data were calculated to evaluate the overall quality of the clean reads. All the downstream analyses were based on the clean data with high quality.

2.6 Mapping analysis of transcriptomic sequencings

Reference genome of mango was downloaded for the RNA-Seq mapping analysis. Version information of the reference genome is Mangifera_indica.v4.0.genome.fa (Wang et al., 2020). HISAT2 (Kim et al., 2019), a Burrows-Wheeler Transform and Ferragina-Manzini (FM) index based search, was used to map clean reads with the reference genome to obtain the localization information of reads on the mango genome. StringTie was applied to assemble the mapped reads for subsequent analysis (Perte et al., 2015). It utilizes a novel network flow algorithm as well as an optional *de novo* assembly step to assemble and quantify transcripts representing multiple spliced variants for each gene locus.

2.7 Analysis of differentially expressed genes (DEGs)

FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) was applied to measure the expression level of a gene or a transcript by StringTie using maximum flow algorithm. In the study, the differential expression genes (DEGs) analysis was performed on comparing the JH4-1 group with the JH24 control group and comparing the JH4-2 group with the JH24 group, respectively. Meanwhile, the DEGs analysis was also performed on comparing the TN4-1 group with the TN24 control group and comparing the JH4-1 group with the JH24 group, respectively. The four comparing groups were named JH24 vs JH4-1, JH24 vs JH4-2, TN24 vs TN4-1, TN24 vs TN4-2.

In the study, transcripts that increased or decreased with a Fold Change (FC) ≥2 and false discovery rate (FDR)<0.01 were considered to be differentially expressed. DESeq2 accepted input of the clean reads and the DEGs between the experimental group and control group were

screened (Liu et al., 2021). The hierarchical clustering map was used to show the distribution of DEGs, and cluster analysis was used to judge the expression pattern of each gene.

2.8 Verification of transcription levels by Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

To confirm gene expression differences of four comparing groups (JH24 vs JH4-1, JH24 vs JH4-2, TN24 vs TN4-1, TN24 vs TN4-2) by transcriptomic sequencing, four DEGs of *probable xyloglucan endotransglucosylase/hydrolase protein 23* (*XTH23*), *probable terpene synthase 12* (*TS12*), *BON1-associated protein 2-like* (*BON1-2*), *RADIALIS-like 4 protein* (*RADIALIS-4*) were selected for qRT-PCR analysis using the primers in Table 1. The amplification program was conducted on a StepOne real-time PCR system (Applied Biosystems) and the cycle condition was as follows: 95℃ for 5 min, followed by 40 cycles of 95℃ for 15 s, and 60℃ for 1 min. The internal control gene, *β-actin*, was used to normalize each gene. Transcription levels of selected DEGs were evaluated based on the $2^{-\Delta\Delta Ct}$ method.

2.9 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of DEGs

GO enrichment analysis of four comparing groups (JH24 vs JH4-1, JH24 vs JH4-2, TN24 vs TN4-1, TN24 vs TN4-2) was conducted by the topGO to analyze the functional classification of DEGs. GO terms were extracted from the best hits obtained from BLASTx against the non-redundant database using Blast2GO (Conesa et al., 2005). Then the obtained DEGs were sorted by GO categories using in-house Perl scripts. GO terms with a *P* value below 0.05 were considered significantly enriched.

Meanwhile, the KEGG database (http://www.genome.jp/kegg/genome) was used to analyze the molecular pathway of these four comparing groups, and DEGs with a *P* value below 0.05 in the KEGG pathways considered to be significantly enriched using ClusterProfiler software (Wu et al., 2021).

3. Results

3.1 The phenotypic and physiological of mango plants under cold stress

The phenotypic observation showed that no obvious symptoms were observed on JH mango plants in JH4-1 group or JH4-2 group, but leaf tips displayed browning after putting back at room temperature (RT) for 3-5 days . Meanwhile, no obvious symptoms were observed on TN

mango plants of TN4-1 group or TN4-2 group, but leaf bases displayed wither and rot at 3-5 days after cold treatment at room temperature.

At physiological levels, JH mango plants performed better than TN mango plants reponses to the low temperature treatment. For instance, comparing TN4-1 group with JH4-1 group, and comparing TN4-2 group with JH4-2 group, the activities of SOD were found to be higher in JH mango plants than that of TN mango plants under cold stress. However, there was no significant difference in SOD activities between the two mango varieties at 24℃ for 72 hrs (Figures 1A). Similar results were observed in the POD activities of the two mango varieties. In detail, the activities of POD were found to be significant differences between the JH mango plants and the TN mango plants at 4℃ treatment for 36 hrs or 4℃ treatment for 72 hrs, while no significant difference of the two groups at 24℃ for 72 hrs (Figures 1B). These findings showed that JH mango is a more cold-tolerant variety than TN and could mitigate the adverse effects of low temperature by increasing the activities of antioxidant defense enzymes.

3.2 Transcriptomic data quality and mapping analysis

The high-quality of 30 total RNA samples from mango leaves (Conc= 150.60 ~ 514.70 ng/μL; $OD_{260/280}$ = 2.01 ~ 2.15; RIN = 8.10 ~ 8.70) were obtained and conformed to the cDNA library preparation requirement. A large number of raw reads were generated from an Illumina NovaSeq 6000 platform and about 20, 000, 000 clean reads of each sample were further generated. In total, the GC content was 42.03 ~ 43.85% and Q30 percentage was over 93.10% for each sample (Table 2), indicating that high quality transcriptomic sequencing clean data was obtained. The clean data of each sample is available in the GenBase in National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences/China National Center for Bioinformation, under accession number CRA016366 that is publicly accessible at https://ngdc.cncb.ac.cn/gsa/browse/CRA016366 (CNCB-NGDC Members and Partners, 2024).

3.3 Screening and cluster analysis of the transcriptomic DEGs

After low temperature treatment, a large number of DEGs were identified in both varieties. For the JH mango variety, 8,337 DEGs (4,051 up-regulated and 4,286 down-regulated) were identified from the JH24 vs JH4-1 using DESeq2 software based on FC and FDR values, while 10,683 DEGs (5,552 up-regulated and 5,131 down-regulated) were further identified in JH24 vs JH4-2, indicating more DEGs were screened when treated at 4℃ for longer (Figure 2 and Table 3). Further analysis revealed that both the highest up-regulated genes were *XTH23*, with log_2FC of 12.93 and 13.93, respectively. The highest down-regulated genes were both of *TS12*, with log_2FC of -11.82 and -10.47, respectively. The qRT-PCR showed the consistent results (Figure 3).

For the TN mango variety, 7,996 DEGs (3,763 up-regulated and 4,233 down-regulated) were identified from TN24 vs TN4-1, while 10,723 DEGs (5,674 up-regulated and 5,049 down-regulated) were further identified in TN24 vs TN4-2, also indicating more DEGs were screened when treated at 4℃ for longer (Figure 2 and Table 3). Further analysis revealed that the highest up-regulated gene was *XTH23* (log_2FC of 13.04) in TN24 vs TN4-1, while it was *BON1-2* (log_2FC of 13.91) in TN24 vs TN4-2. The highest down-regulated genes were both of *RADIALIS-4*, with log_2FC of -11.03 and -10.97, respectively. This outcome was also confirmed by the qRT-PCR (Figure 3).

3.4 GO and KEGG enrichment analyses of DEGs

Functional analysis of the DEGs showed that 31 GO terms (q value < 0.05) were annotated in JH24 vs JH4-1, and nine of them associated with photosynthesis or chloroplast. Furthermore, 62.5% (10/16) of the GO terms were annotated into photosynthesis or chloroplast pathways in JH24 vs JH4-2 when JH treated at 4℃ for longer. In TN24 vs TN4-1, nine of them (9/46, 19.6%), the same GO terms as JH24 vs JH4-1, were annotated. In addition, eight (8/16, 50.0%) photosynthesis or chloroplast associated GO terms were identified when TN treated at 4℃ for longer (Figure 4 and Figure 5).

In general, more KEGG pathways were enriched and annotated when two varities treated at low temperature longer. KEGG enrichment annotation analysis was performed on DEGs from different comparison groups of JH24 vs JH4-1, JH24 vs JH4-2, TN24 vs TN4-1, and TN24 vs TN4-2. The results showed that 132, 134, 131 and 133 KEGG pathways were annotated, respectively (Figure 6). In detail, three significant KEGG pathways were found in JH24 vs JH4-1 by enrichment analysis, namely photosynthesis-antenna proteins KEGG, photosynthesis KEGG, and valine, leucine and isoleucine degradation KEGG. In JH24 vs JH4-2, in addition to annotating to these three pathways, four KEGGs of thiamine metabolism KEGG, inositol phosphate metabolism KEGG, glycine, serine and threonine metabolism KEGG, and glyoxylate and dicarboxylate metabolism KEGG pathways were annotated. Similarly, three enrichment pathways were found in TN24 vs TN4-1, including photosynthesis KEGG, photosynthesis-antenna proteins KEGG and proteasome KEGG. However, more enrichment pathways were found in TN24 vs TN4-2, including oxidative phosphorylation KEGG, ubiquitin mediated proteolysis KEGG and inositol phosphate metabolism KEGG pathways when treated at 4℃ for longer (Figure 7).

3.5 Common and specific cold resistance pathways

Two common KEGG pathways, photosynthesis-antenna proteins and photosynthesis pathways, were found by comparing the KEGG enrichments from different comparison groups of JH24 vs JH4-1, JH24 vs JH4-2, TN24 vs TN4-1, and TN24 vs TN4-2. Further analysis of the annotated

DEGs showed that there was the same up-down gene mode pattern in the pathways, suggesting that these two pathways are the primary protection response pathways to cold resistance in mango (Supplementary Figure 1 and Supplementary Figure 2). However, in addition to these two common pathways, it was found that the cold-resistant JH mango was annotated to a meaningful pathway, valine, leucine and isoleucine degradation pathway when treated with low temperature for 36 hrs. Furthermore, when treated with low temperature for 72 hrs, more specific pathways were identified and obtained in JH, such as thiamine metabolism pathway, inositol phosphate metabolism pathway, glycine, serine and threonine metabolism pathway, and glyoxylate and dicarboxylate metabolism pathway (Table 4), indicating that this is the specific cold resistance regulation pathway of cold tolerant mango varieties.

4. Discussion

The key step of identification and evaluation of plant cold resistance is to obtain cold tolerant and non-cold tolerant experimental materials. Then, the important DEGs and signaling pathways were found by high-throughput transcriptomics. Before carrying out this experiment, more mango varieties were selected for cold resistance test, such as TN, JH, Guifei, Hongyu, Xiangya, Jidan varieties etc. The results showed that a cold tolerant mango variety JH and a cold sensitive mango variety TN were selected. We used these two mango varieties as experimental subjects to study the DEGs and difference KEGG pathways under cold stress and identification of specific cold resistance regulation pathways through the combination of transcriptome high-throughput sequencing and molecular biology.

In order to further clarify the ability of growing in low temperature of two mango varieties JH and TN, we evaluated their cold resistance by phenotype and physiological and biochemical indexes after cold treatment. Although no obvious symptoms were observation in JH and TN when treated at 4℃ for 36 and 72 hrs, but leaf browning, necrosis and other cold damage phenotypes were observed when putback in RT for 3-5 days, and leaf rot occurred when place in RT more time. However, SOD and POD activities *in vivo* were real-time changed to varying degrees at 36 and 72 hrs after treatment at 4℃. These results indicated that the physiological and biochemical results of mango plants were responsive in time when treated at low temperature, but the symptom phenotype was delayed.

The expression of *SOD* and *POD* genes in plants is controlled by various environmental stresses, which are important protective enzymes in plants. They can reflect the changes of metabolism and stress resistance in plants at a certain period. The results of this study showed that the SOD and POD activities of TN and JH were changed in different degrees when treated at low temperature, and the SOD activity decreased at two time points of low temperature treatment, while the POD activity increased. As a cell membrane protective enzyme, SOD plays a role

when the temperature changes, and too high or too low temperature will affect the membrane system, resulting in the destruction of proteins and DNA in the cell (Saed-Moucheshi et al., 2021). Some reports prove that SOD activity is positively correlated with plant cold resistance (Li et al., 2024), while others prove that they are negatively correlated (Raza et al., 2021). In this study, the SOD activity in mango leaves showed a downward trend after low temperature stress, which may be because the expression of SOD enzyme gene in mango is inhibited at low temperature, resulting in the decrease of unit tissue enzyme content. Therefore, after low temperature stimulation, although the activity of unit enzyme increased, the overall activity of SOD in mango leaves decreased due to the influence of the expression level. Interestingly, SOD activity of JH was significantly higher than that of TN, indicating that cold resistant varieties increased SOD activity more rapidly than non-cold resistant varieties under low temperature stimulation.

POD activity increased at both time points of low temperature treatment. POD is one of the key enzymes in the enzymatic defense system in plants under stress conditions. It works synergistically with SOD and catalase (CAT) to remove excess free radicals in the body, thereby improving plant stress resistance (Gao et al., 2024). In this study, the POD activity of the leaves of the two mango varieties under low temperature stress showed an increasing trend, and the increase of SOD activity of the cold resistant mango JH was significantly greater than that of the non-cold resistant mango variety TN, indicating that POD activity was positively correlated with the cold resistance of mango.

In this study, a large amount of DEGs were obtained from JH and TN mango plants, and KEGG annotated two common pathways in response to low temperature induction, namely, the photosynthesis-antenna proteins pathway and photosynthesis pathways. Further, it was found that there was no significant difference in the annotated DEGs species and up-/down-regulated genes in these two pathways, indicating that these are basic cold resistance pathways of different mango varieties in response to low temperature induction, and play an important role in the primary cold resistance pathway of plants. Studies have shown that *Rhododendron* plants were treated at low temperatures, and the photosynthesis-antenna proteins and photosynthesis pathways were activated (Liu et al., 2020). Photosynthesis is a crucial process in plants that converts light energy into chemical energy, enabling them to produce carbohydrates and sustain growth. However, under cold stress, photosynthesis can be severely impaired. The photosynthesis–antenna proteins pathway plays a key role in optimizing the efficiency of photosynthetic light harvesting. This allows the plant to balance energy absorption from light and prevents excess energy from causing photodamage (Dhawi, 2024). By maintaining a balanced antenna protein system, mango plants can continue to perform photosynthesis, albeit at a reduced rate, under stressful conditions.

In this study, the KEGG enrichment analyses of DEGs correlated with amino acid and carbohydrate metabolisms were significantly annotated. As previous studies showed, amino acid and carbohydrate metabolic pathways play key roles in the cold stress tolerance in plants (Zhang et al., 2017). The three essential amino acid metabolisms, such as valine, leucine and isoleucine, are well thought-out in mango metabolisms. Similar observations have also been detected in a combined metabolome and phenome analysis of plants under cold stress conditions (Hildebrandt, 2018). To date, many combined-omics studies have detected the vital role of these metabolic pathways, such as in *Nicotiana tabacum* leaves under cold stress condition (Song et al., 2024), in tomato under salt stress condition (Zhang et al., 2017), in soybean under salinity stress condition (Qian et al., 2019), and in switchgrass under drought and heat stresses conditions (Ayyappan et al., 2024). Thus, these discoveries advised that cold stress tolerance of mango could be related to normalizing amino acid accumulation and/or a breakdown in valine, leucine and isoleucine metabolism.

The inositol phosphate metabolism is one of the major enriched biological pathways detected during cold stress, mainly in JH. Inositol phosphate synthase is the key enzyme of inositol synthesis, which is a central molecule required for cell metabolism and plant growth as a precursor to a large variety of compounds. As previous studies showed, inositol phosphate metabolism was induced in leaflets of M. *falcata* under cold and dehydrant stress to confer multiple resistances to abiotic stresses (Tan et al., 2013). Nevertheless, their significant roles in cold stress tolerance in different crop plants needs more investigation.

In addition, the researchers also found that the expression of some genes and proteins is closely related to the cold resistance of mango, such as *putative calcium-binding protein CML19*, with $\log_2$FC of 13.52 in the study. Some studies have been showed that calmodulin-like (CML) proteins are major calcium sensors that play a critical role in cold stimulus response in plants (Aleynova et al., 2023; Wu et al., 2023).

It is expected to cultivate mango varieties with stronger cold resistance by regulating the expression of these genes through genetic engineering. In summary, these studies provide an important theoretical and practical basis for improving the cold resistance and adaptability of mango, and help to promote the sustainable development of mango industry.

5. Conclusions

In the study, SOD and POD activity measurements showed that Jinhuang (JH) mango variety were found to be more cold resistance than Tainong (TN) mango activity. Transcriptomics showed two primary cold resistant regulation pathways, photosynthesis-antenna proteins

pathway and photosynthesis pathway, were both significant annotated in the two mango varieties, indicating share the common regulation mechanism response to cold stress. Furthermore, five specific cold resistant pathways, such as amino acid and carbohydrate metabolisms, were identified in JH mango variety, indicating the specific regulation pathways in cold tolerant mango varieties. These results provided insights into the primary and specific molecular mechanisms of different mango variety resistance to chill.

ADDITIONAL INFORMATION AND DECLARATIONS

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Author Contributions

J.H.W, X.Y.F, and M.A.A performed the experiments; N.T.Y conceived and designed the experiments; Y.F.L, Y.K, Q.L.C, S.L.X, H.X.Y, and N.T.Y analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Data Availability

The clean data of 20 mango transcriptomic sequencings is available in the GenBase in National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences/China National Center for Bioinformation, under accession number CRA016366 that is publicly accessible at https://ngdc.cncb.ac.cn/gsa/browse/CRA016366.

Supplementary Information

Supplemental information for this article can be found online at : XXX.

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Table 1(on next page)

Table 1. The list of primers in this study.

1 **Table 1.** The list of primers in this study.

2

Primer name	Primer sequence (5'-3')	Usage	Length
adpter3	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC	Adaptor primers	/
adpter5	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT	for libraries	
β-actin-qF	GAATATGAAACTGCCCCTTGC	RT-qPCR	109bp
β-actin-qR	CTTCCCGAAATAGACCTGATCC		
XTH23-qF	CGCCTTCACTCCCACTATAATC	RT-qPCR	177bp
XTH23-qR	GCCATCTCCCCAAGTGATATC		
TS12-qF	AGACACCATCCACAAAGAGC	RT-qPCR	189bp
TS12-qR	CTTTTCCCTGTTCTCGCATTC		
RADIALIS-4-qF	ATTCCTTTCCCCATCTCTTCG	RT-qPCR	136bp
RADIALIS-4-qR	AAGAAAGGTGGAGTTGTGGAG		
BON1-2-qF	CAGTGAGAGTAAATACGCCAGG	RT-qPCR	134bp
BON1-2-qR	CCATGAACAAGACCCATATCCC		

Table 2(on next page)

Table 2. Summary of the transcriptome data from mango leaves of cold resistant variety (Jinhuang, JH) and non-cold resistant variety (Tainong, TN) with low temperatures treatment.

Table 2. Summary of the transcriptome data from mango leaves of cold resistant variety (Jinhuang, JH) and non-cold resistant variety (Tainong, TN) with low temperatures treatment.

Group	Samples	Clean reads	Clean bases	GC content	%≥Q30	Treatment
JH24	JH24-1	20,690,998	6,191,931,316	43.81%	94.04%	Treatment at 24°C for 72 hours
	JH24-2	22,740,155	6,802,674,662	43.85%	95.87%	
	JH24-3	26,627,548	7,963,214,582	43.56%	94.11%	
	JH24-4	19,360,036	5,794,480,296	43.61%	93.89%	
	JH24-5	20,722,438	6,201,795,334	43.69%	94.05%	
JH4-1	JH4-1-1	20,859,908	6,238,506,022	43.23%	94.04%	Treatment at 4° C for 36 hours
	JH4-1-2	19,727,979	5,900,217,082	42.72%	93.93%	
	JH4-1-3	22,612,265	6,763,548,252	43.39%	96.38%	
	JH4-1-4	20,939,664	6,263,632,400	42.54%	94.33%	
	JH4-1-5	22,126,488	6,618,948,418	43.24%	96.04%	
JH4-2	JH4-2-1	20,794,165	6,221,499,546	42.66%	94.38%	Treatment at 4° C for 72 hours
	JH4-2-2	21,067,749	6,298,223,294	42.03%	95.83%	
	JH4-2-3	20,303,738	6,072,163,702	42.98%	93.91%	
	JH4-2-4	21,356,777	6,376,778,350	43.06%	94.15%	
	JH4-2-5	20,885,262	6,246,790,516	43.37%	94.03%	
TN24	TN24-1	19,179,465	5,741,047,294	43.52%	93.10%	Treatment at 24° C for 72 hours
	TN24-2	19,539,704	5,846,807,346	43.51%	93.37%	
	TN24-3	22,585,139	6,749,521,950	43.29%	93.79%	
	TN24-4	23,735,944	7,095,142,634	43.62%	94.54%	
	TN24-5	23,910,654	7,157,151,728	43.30%	93.75%	
TN4-1	TN4-1-1	20,707,435	6,193,858,750	42.96%	93.79%	Treatment at 4° C f

	TN4-1-2	23,302,615	6,950,245,902	43.16%	94.55%	
	TN4-1-3	22,389,472	6,681,149,224	43.51%	94.13%	
	TN4-1-4	20,855,891	6,236,494,380	43.30%	94.45%	
	TN4-1-5	22,463,201	6,717,550,880	43.00%	95.94%	
	TN4-2-1	23,419,329	6,998,287,042	42.35%	93.96%	
	TN4-2-2	20,533,795	6,140,930,166	42.10%	93.54%	Treatment at 4° C for 72 hours
TN4-2	TN4-2-3	19,563,471	5,851,295,024	42.04%	93.28%	
	TN4-2-4	20,426,507	6,104,773,202	42.38%	94.03%	
	TN4-2-5	20,547,675	6,142,268,260	42.10%	93.69%	

3

Table 3(on next page)

Table 3. Statistics of DEGs between low temperature (4℃) treatment group and control (24℃) group in different mango varieties.

Table 3. Statistics of DEGs between low temperature (4℃) treatment group and control (24℃) group in different mango varieties.

Group	DEG Number	NR	GO	KEGG	Up-regulated	Down-regulated
JH24 vs JH4-1	8,337	8,030	6,764	5,748	4,051	4,286
JH24 vs JH4-2	10,683	10,212	8,533	7,258	5,552	5,131
TN24 vs TN4-1	7,996	7,714	6,517	5,595	3,763	4,233
TN24 vs TN4-2	10,723	10,312	8,696	7,406	5,674	5,049

Table 4(on next page)

Table 4. The list of KEGG enrichment pathways between 4℃ treatment group and control group in different mango varieties.

Table 4. The list of KEGG enrichment pathways between 4℃ treatment group and control group in different mango varieties.

Groups comparison	KEGG B Class	KEGG ID	Pathway	q value	Gene number
JH24 vs JH4-1	Energy metabolism	ko00196	Photosynthesis - antenna proteins	2.40E-11	22
	Energy metabolism	ko00195	Photosynthesis	8.81E-11	49
	Amino acid metabolism	ko00280	Valine, leucine and isoleucine degradation①	2.11E-03	51
JH24 vs JH4-2	Energy metabolism	ko00195	Photosynthesis	1.51E-09	54
	Energy metabolism	ko00196	Photosynthesis - antenna proteins	2.36E-09	22
	Amino acid metabolism	ko00280	Valine, leucine and isoleucine degradation①	6.95E-03	59
	Metabolism of cofactors and vitamins	ko00730	Thiamine metabolism①	8.54E-03	29
	Carbohydrate metabolism	ko00562	Inositol phosphate metabolism①	9.16E-03	75
	Amino acid metabolism	ko00260	Glycine, serine and threonine metabolism①	1.39E-02	54
	Carbohydrate metabolism	ko00630	Glyoxylate and dicarboxylate metabolism①	1.55E-02	65
TN24 vs TN4-1	Energy metabolism	ko00195	Photosynthesis	2.17E-05	40
	Energy metabolism	ko00196	Photosynthesis - antenna proteins	1.29E-04	16
	Folding, sorting and degradation	ko03050	Proteasome	2.93E-02	38
TN24 vs TN4-2	Energy metabolism	ko00196	Photosynthesis - antenna proteins	2.72E-06	20
	Energy metabolism	ko00195	Photosynthesis	1.58E-05	47
	Folding, sorting and degradation	ko03050	Proteasome	1.16E-02	48
	Energy metabolism	ko00190	Oxidative phosphorylation	1.18E-02	85
	Folding, sorting and degradation	ko04120	Ubiquitin mediated proteolysis	1.70E-02	128
	Carbohydrate metabolism	ko00562	Inositol phosphate metabolism	3.38E-02	73

① Specific KEGG pathways resistance to cold stress in JH mango varieties.

Figure 1

Figure 1. Physiological responses of JH and TN mango varieties responding to cold stress (4℃ for 36 hrs or 4℃ for 72 hrs) and untreated group (24℃ for 72 hrs)

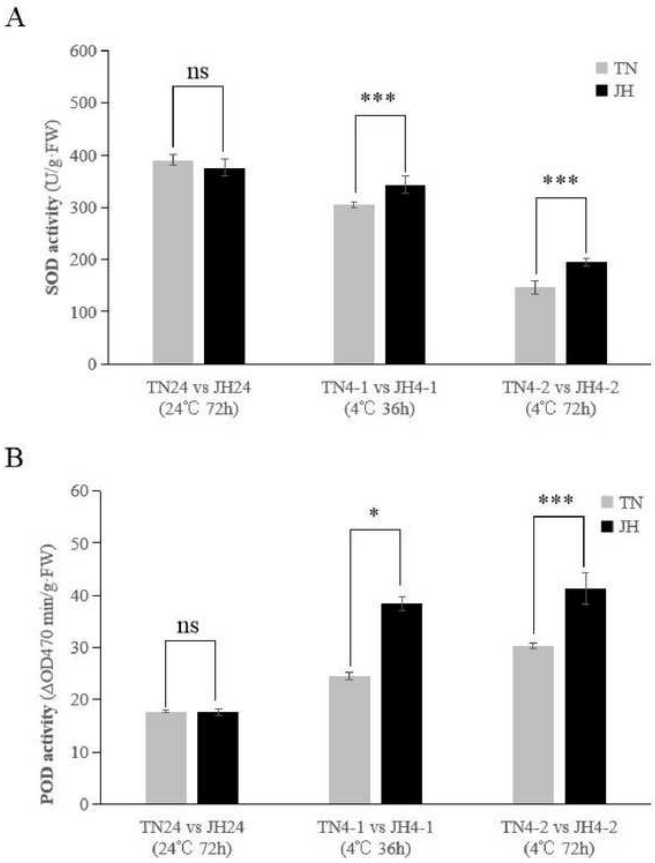

Figure 1. Physiological responses of JH and TN mango varieties responding to cold stress (4℃ for 36 hrs or 4℃ for 72 hrs) and untreated group (24℃ for 72 hrs). (A) peroxidase (POD) activity, (B) superoxide dismutase (SOD) activity. The statistical significance was determined via a two-way ANOVA and Dunnett's multiple comparisons test with ***$p \leq 0.01$, *$p \leq 0.05$, and ns mean non-significant.

Figure 2

Figure 2. Volcano plot of DEGs between low temperature treatment group and control group in different mango varieties.

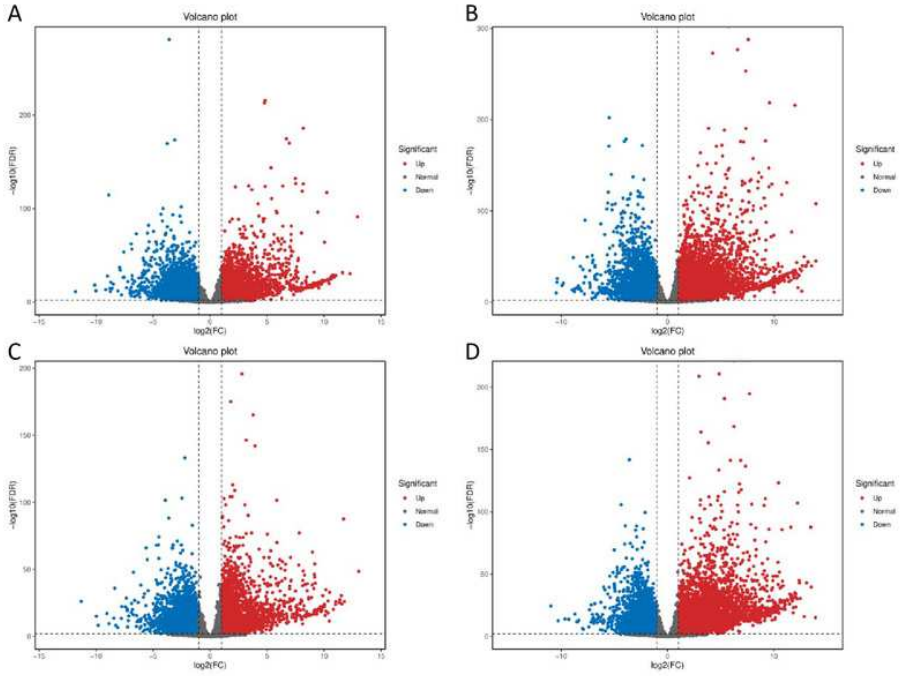

Figure 2. Volcano plot of DEGs between low temperature treatment group and control group in different mango varieties. (A) JH24 group vs JH4-1 group, (B) JH24 group vs JH4-2 group, (C) TN24 group vs TN4-1 group, (D) TN24 group vs TN4-2 group. The abscissa $\log_2$(FC) represents the fold change, while the ordinate -$\log_{10}$(FDR) represents the false discovery rate. In the study, $\log_2$(FC)≥2 and -$\log_{10}$(FDR)<0.01 indicates the differentially expressed genes.

Figure 3

Figure 3. Comparison of the transcription levels of selected DEGs between qRT-PCR (blue) and Illumina NovaSeq 6000 sequencing (red).

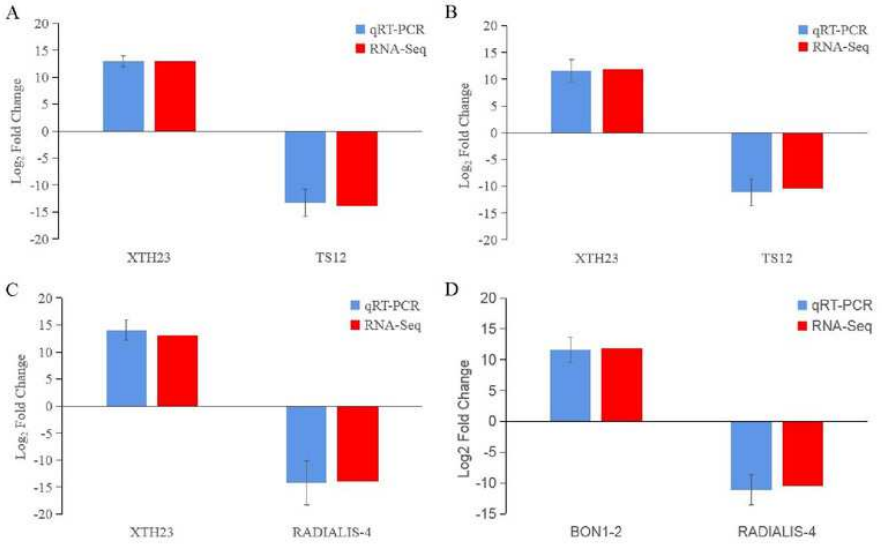

Figure 3. Comparison of the transcription levels of selected DEGs between qRT-PCR (blue) and Illumina NovaSeq 6000 sequencing (red). (A) JH24 group vs JH4-1 group, (B) JH24 group vs JH4-2 group, (C) TN24 group vs TN4-1 group, (D) TN24 group vs TN4-2 group. Target gene abbreviations are as follows: *probable xyloglucan endotransglucosylase/hydrolase protein 23* (*XTH23*), *probable terpene synthase 12* (*TS12*), *BON1-associated protein 2-like* (*BON1-2*), *RADIALIS-like 4 protein* (*RADIALIS-4*). The internal control gene, *β-actin*, was used to normalize each gene. Transcription levels of selected DEGs were evaluated based on the $2^{-\triangle\triangle Ct}$ method. Error bars indicated standard deviations of averages from three replicates. Value above and below the abscissa represent up-regulation and down-regulation, respectively.

Figure 4

Figure 4. Statistics of GO annotation classification of DEGs between low temperature treatment group and control group in different mango varieties.

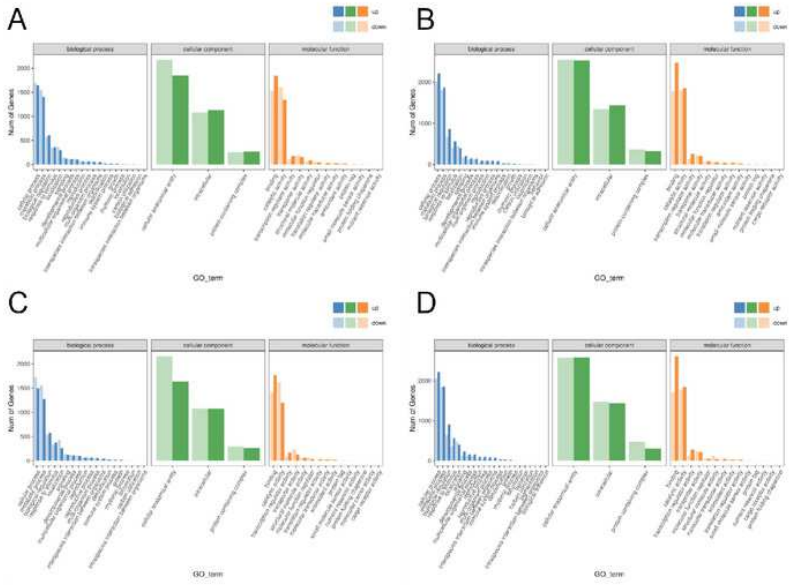

Figure 4. Statistics of GO annotation classification of DEGs between low temperature treatment group and control group in different mango varieties. (A) JH24 group vs JH4-1 group, (B) JH24 group vs JH4-2 group, (C) TN24 group vs TN4-1 group, (D) TN24 group vs TN4-2 group.

Figure 5

Figure 5. Histogram of GO enrichment annotation of DEGs between low temperature treatment group and control group in different mango varieties.

Figure 5. Histogram of GO enrichment annotation of DEGs between low temperature treatment group and control group in different mango varieties. (A) Biological process of JH24 group vs JH4-1 group, (B) Cellular component of JH24 group vs JH4-1 group, (C) Molecular function of JH24 group vs JH4-1 group, (D) Biological process of JH24 group vs JH4-2 group, (E) Cellular component of JH24 group vs JH4-2 group, (F) Molecular function of JH24 group vs JH4-2 group, (G) Biological process of TN24 group vs TN4-1 group, (H) Cellular component of TN24 group vs TN4-1 group, (I) Molecular function of TN24 group vs TN4-1 group, (J) Biological process of TN24 group vs TN4-2 group, (K) Cellular component of TN24 group vs TN4-2 group, (L) Molecular function of TN24 group vs TN4-2 group.

Figure 6

Figure 6. Statistics of KEGG annotation classification of DEGs between low temperature treatment group and control group in different mango varieties.

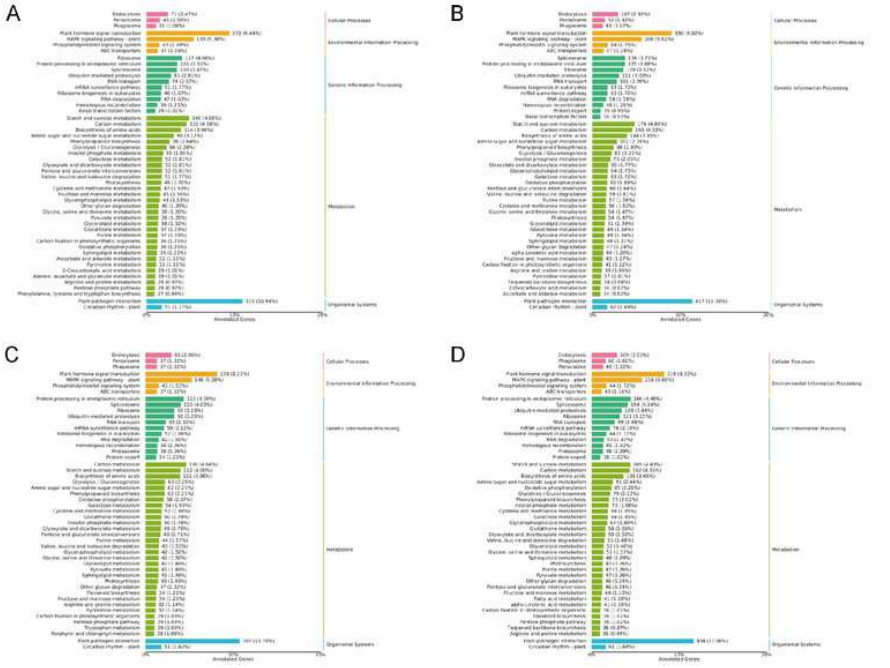

Figure 6. Statistics of KEGG annotation classification of DEGs between low temperature treatment group and control group in different mango varieties. (A) JH24 group vs JH4-1 group, (B) JH24 group vs JH4-2 group, (C) TN24 group vs TN4-1 group, (D) TN24 group vs TN4-2 group

Figure 7

Figure 7. Bubble chart of KEGG enrichment pathways between low temperature treatment group and control group in different mango varieties.

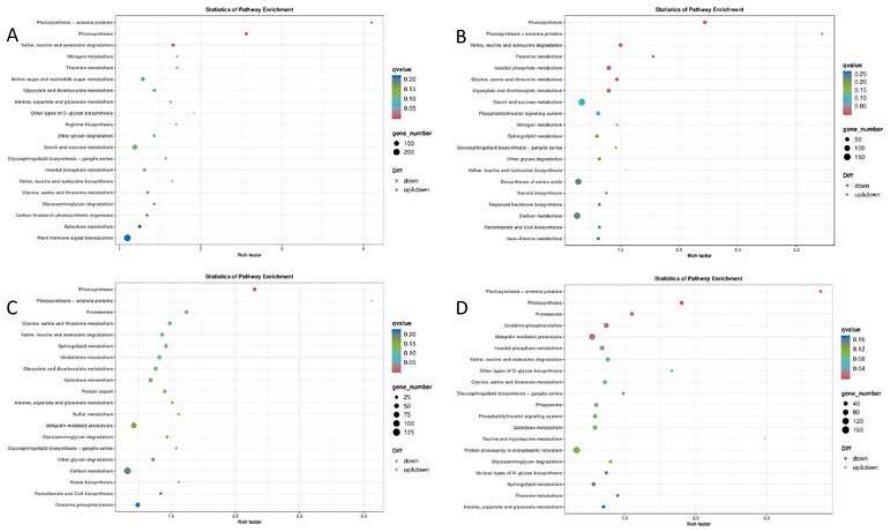

Figure 7. Bubble chart of KEGG enrichment pathways between low temperature treatment group and control group in different mango varieties. (A) JH24 group vs JH4-1 group, (B) JH24 group vs JH4-2 group, (C) TN24 group vs TN4-1 group, (D) TN24 group vs TN4-2 group. Note: Each dot represents a KEGG pathway. Y-axis: Pathway; X-axis: Rich factor.